Examining the Distribution of Ketamine and Metabolites in Decomposed Skeletal Tissues: Development of a High Throughput Approach and Application to an Environmental and Decomposition Study

by

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Abstract

The use of skeletal tissues in forensic toxicology research has increased dramatically in the last 5 years. These studies have yielded valuable information pertinent to using skeletal tissues as a biological matrix in drug analysis. The majority of these studies have been completed on a small scale so a larger scale is needed for further research. Here, a high throughput microwave assisted extraction (MAE) and microplate solid phase extraction (MPSPE) protocol is developed and implemented to examine how body position and microclimate affect ketamine and metabolite distribution in decomposed skeletal tissue. Analytes were successful recovered in 30 min following MAE compared to 180 min for passive solvent extraction (PSE). Body proved to be significantly different in the observed drug levels at both microclimates. The sunlit microclimate had significantly higher drug levels, with less maggot activity, compared to the shaded microclimate.

Keywords

Forensic Toxicology, Bone, Ketamine, GC-MS, Microwave Assisted Extraction, Decomposition

Co-Authorship Statement

Chapter 1 of this thesis reviews many of technological and chemical concepts relevant to this project. I am the sole author of this chapter. James Watterson contributed to experimental design and concepts, along with structural guidance through this thesis.

Chapter 2 is a restructured document of a published article in Analytical Methods entitled, Microwave assisted extraction of ketamine and metabolites in skeletal tissues (*Anal. Methods*, 2014, 6 (4), 1142-1148). I am the first author and James Watterson is the second author. Chapter 2 also contains additional data, describing passive solvent extraction, solid phase extraction optimization, gas chromatography mass spectrometry optimization and particle size comparison.

Chapter 3 is a manuscript that has been submitted to the Journal of Analytical Toxicology and is under peer-review. I am the first author on this manuscript and James Watterson is the second author.

Chapter 4 discusses conclusion of this thesis and suggests avenues for future research in this area. I am the sole author with conceptual guidance from James Watterson.

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List of Abbreviations

Abbreviation	Definition
ACE:HEX	Acetone:Hexane
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
BTE	Bone tissue extract
˚C	Degrees Celsius
%CV	Coefficient of Variation
CO2	Carbon dioxide
CVRT	Cervical vertebra
%DA	Percent decrease in absorbance
deg/min	Degrees per minute
DHNK	Dehydronorketamine
e.g.	*Exempli gratia*
EI	Electron impact ionization
ESI	Electrospray ionization
et al.	*et alia*
etc.	*et cetera*
g	Gram
GC	Gas chromatography
GC-FID	Gas chromatography-Flame ionization detection
GC-MS	Gas chromatography-Mass spectrometry
GC-NPD	Gas chromatography-Nitrogen phosphorus
GHz	Gigahertz
HPLC	High performance liquid chromatography
HUM	Humerus
i.e.	*id est*
i.p.	intraperitonally
KET	Ketamine
KET-d4	Ketamine-deuterated
kg	Kilogram
KW	Kruskall-Wallis
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LoD	Limit of Detection
LoQ	Limit of Quantification
LVRT	Lumbar Vertebra
MAE	Microwave assisted extraction
MAX/MIN	Maximum value over minimum value ratio

mg	Milligram
min	Minute
mL	Millilitre
mmHg	Millimeters of mercury
MPSPE	Microplate solid phase extraction
MS	Mass spectrometry
NDMA	N-methyl-D-aspartate
ng	Nanogram
NKET	Norketamine
NKET-d4	norketamine-deuterated
PB6	Phosphate buffer saline (pH 6.0, 0.1 M)
PEL	Pelvis
PICI	Positive ion chemical ionization
pKa	Acid dissociation constant
PRO	Prone
PSE	Passive solvent extraction
PTFE	Polytetrafluoroethylene
R^2	Linearity
RIB	Rib
rpm	Revolutions per minute
RR	Response ratio
RR/m	Mass normalized response ratio
$RR_{metabolite}$/RRparent	Ratio of levels of metabolite and parent drug
$RR_{parent}/RR_{metabolite}$	Ratio of levels of parent drug and metabolite
SCAP	Scapula
SIM	Selected ion monitoring
SPE	Solid phase extraction
SUP	Supine
SWGTOX	Scientific working group for forensic toxicology
TIB	Tibia
TOF	Time of flight
TVRT	Thoracic vertebra
µg	Microgram
UPR	Upright
v/v	Volume per volume
W	Watt

Chapter 1

1 Introduction

1.1 Forensic Toxicology

Forensic toxicology is a branch of forensic science that studies the effects of chemicals on living organisms to aid in legal investigations (Levine, 2010). It encompasses principles from other disciplines including chemistry, biology and pharmacology. Forensic toxicology consists of three subfields: post-mortem toxicology, human performance toxicology and forensic drug testing (Levine, 2010). Post-mortem toxicology is used to aid in the determination of cause of death and to diagnosis drug intoxications. Blood is the most commonly analyzed specimen since drug levels can be linked to non-fatal and fatal concentrations. Other specimens used for analysis include: urine, hair, liver, vitreous humor, bile and muscle. Skeletal tissues, bone and bone marrow, are not typically used in post-mortem analysis; there is no database to correlate drug levels with intoxication. In cases of severe decomposition, bone tissue may be the only specimen available for collection so it is important to access its potential in post-mortem analyses.

1.2 Bone Physiology and Drug Deposition in Bone

The extracellular matrix of bone is composed largely of inorganic materials; the most abundant being hydroxyapatite (Ca_{10} $(PO_4)_6OH_2$) (Martini et al., 2009). Other inorganic materials include salts (calcium carbonate) and ions (sodium, magnesium and fluoride), while the major organic material are collagen fibers. The hydroxyapatite crystals form on collagen fibers which provide a strong framework for the bone (Martini et al., 2009). Osteocytes are the most abundant bone cell type and are responsible for maintaining mineral and protein content for the extracellular matrix, as well as repairing damaged bone (Martini et al., 2009). Osteoblasts (cells that build bone matrix), osteoclasts (cells that remove and recycle bone matrix) and osteoprogenitor cells (cells that produces osteoblasts) are also present. Osteocytes are located in lacunae (pockets layered between lamellae). Canaliculi (canals) connect lacunae, through the lamellae, and create a pathway to transfer materials between cells (Martini et al., 2009).

Compact bone is composed of osteons (Haversian system). Within an osteon, osteocytes are arranged in coaxial layer around the Haversian canal (central canal) which runs parallel with the

length of the bone (Martini et al., 2009). Perforating canals run perpendicular to the bone surface. These canals are responsible for transporting materials to the bone. Spongy bone contains struts and plates, termed trabeculae, which contain lamellae, osteocytes, lacunae and canaliculi (Martini et al., 2009). The trabeculae are adjacent to cavities that contain red bone marrow. The canaliculi open onto the surface of the trabeculae and allow the transfer of materials to the osteocytes. Short (i.e. carpals) and irregular bones (i.e. Vertebra) contain a thin layer of compact bone surround centrally located spongy bone. Long bones (i.e. femur, tibia, and humerus) contain compact bone in the diaphysis (midsection of the bone) and spongy bone in the epiphysis (rounded end of the bone). Flat bones (i.e. ribs, scapula, and pelvis) contain two thin layers of compact bone surrounding spongy bone.

The degree of drug deposition into bone is heavily influenced by the structural and physiological features of the various bones. Differences in bone architecture provide varying degrees of surface area for the drugs to make contact (Stempemsky et al., 2003). The various bones have unique compositions of cortical and cancellous bone which may provide storage sites for drugs. The type of bone marrow (red or yellow) may provide storage sites based upon lipophilicity of the drug. These considerations have to lead to three potential mechanism of drug deposition into bone. The first mechanism involves the absorption of the drug by the bloodstream into systemic circulation, and the subsequent rapid uptake of drugs by the canaliculi which transport them to the lacunae (Stepemsky et al., 2003). The second mechanism involves drug transport from systemic circulation to the bone marrow. Bone marrow has a rich vascularization, high lipid content and encasement within the bone which make it a potential repository for drugs (Cartiser et al., 2011). The third mechanism is thought to involve the process of decomposition. During decomposition, soft tissues liquefy which results in drug partitioning. The drug-rich fluid may then be absorbed by the surrounding bone due to its somewhat porous nature. Additional factors that affect drug deposition into bone include: route of administration, distribution at time of death and physiochemical characteristics of the drug.

1.3 Drug Isolation and Preparation Techniques in Bone

1.3.1 Passive Solvent Extraction

Passive solvent extraction (PSE) is based upon diffusion between a solid matrix and a liquid solvent. In PSE, heat is transferred to the vessel containing the matrix. The solvent is

heated which causes desorption of constituents from the matrix. These constituents diffuse into and become dissolved in the bulk solvent. The supernatant is collected and assayed. Parameters often influencing PSE include: extraction time, choice of solvent, solvent volume, temperature, mass and particle size. Table 1 lists the factors that affect the extraction efficiency of PSE.

1.3.2 Microwave Assisted Extraction

The first publications involving extraction of organic compounds using microwave assisted extraction (MAE) was published in 1986 by Ganzler, Salgo and Valko (cited in Letellier et al., 1999). In these studies, the authors developed extraction protocols for lipids, antinutritives and pesticides in different matrices; soils, seeds, foods and feeds. More recent studies have examined the application of MAE for soils, sediments and hair (Bartolomé et al., 2005; Liu et al., 2004; Fernández et al., 2009). Microwaves are a form of electromagnetic radiation, ranging from 300 MHZ to 300 GHz, composed of oscillating perpendicular magnetic and electric fields (Mandal et al., 2007). They are used as both information carriers and energy vectors which transform electromagnetic energy into heat as a result of wave adsorption (Mandal et al., 2007). Commercial microwaves operate at a frequency of 2450 MHz (Letellier et al., 1999).

In MAE, microwave radiation is absorbed by the solvent and/or sample, which leads to sample heating by two mechanisms: ionic conduction and/or dipole rotation, which usually occur simultaneously (Mandal et al., 2007). Ionic conduction occurs as a result of ion migration under a changing electric field (Mandal et al., 2007). The resistance by the solution to the movement of ions generates friction which, in turn, heats the solution. Dipole rotation involves the rearrangement of the molecular dipoles under the influence of the oscillating electric filed. At a frequency of 2450 MHz, the dipoles align and realign 4.9×10^4 times per second (Letellier et al., 1999). This rapid movement prevents the solvent molecules from aligning with the electric field. The molecules start to vibrate and produce friction which results in heating. Greater than 2450 MHz, the molecules do not get a chance to start aligning with the electric field, due to an even more rapid speed, so no heating occurs (Letellier et al., 1999). At a frequency of less than 2450 MHz, the molecules are able to align with the electric filed so no heating occurs (Letellier et al., 1999). These mechanisms allow for direct localized heating at the surface of the sample and result in desorption of the analytes and dissolution in the solvent.

The efficiency of solvent heating under microwave irradiation is a function of the dissipation factor (tan δ). The dissipation factor is displayed by the following equation:

$$\tan \delta = \varepsilon'' / \varepsilon' \text{ (Letellier et al., 1999)}$$

where ε'' is the dielectric loss factor which describes the efficiency of converting microwave energy into heat and ε' is the dielectric constant which indicates the solvents ability to absorb microwave energy. Polar compounds (i.e. methanol and ethanol) absorb microwave energy with a greater efficiency due to the presence of a permanent dipole (greater dielectric loss factor) (Letellier et al., 1999). Parameters often investigated in MAE include: extraction time, choice of solvent, solvent volume, temperature, mass, power and particle size. Table 1 lists the factors that affect the extraction efficiency of PSE and MAE.

Table 1: Factors affecting the extraction efficiency of PSE and MAE

Parameter	PSE	MAE
Extraction Time	Solvent, Matrix	Solvent, Matrix
Particle Size	Matrix composition	Matrix composition
Power	N/A	Number of samples, Solvent, Matrix
Solvent	Dissolution ability, Stability	Absorption ability, Stability
Solvent Volume	Mass	Mass
Temperature	Stability, Solvent	Stability, Solvent

1.3.3 Bone Preparation

Multiple bone preparation techniques have been used to analyze drugs in bone tissue. Table 2 summarizes some of the preparation methods and the drug isolation techniques used. Passive solvent extraction is the most frequently used method to isolate drugs from bone tissue and uses a variety of bone preparation techniques. Horak et al. directly compared two different bone preparation techniques using passive solvent extraction to detect citalopram and olanzapine. The authors incubated full bone and bone silvers in both methanol and water (Horak et al., 2007).

Citalopram was the only drug present and it gave the highest signal from bone slivers that were extracted in methanol and the lowest signals were achieved from whole bone extracted with water (Horak et al., 2007). Ground bone samples have been used for all MAE work completed to date by Watterson and collaborators. Acid digestion has been used in two studies to isolate drugs from bone matrix (Raikos et al., 2001; Guillot et al., 2007). In both these studies, samples were ground prior to digestion.

Drummer (2008) published a review that highlighted advantages and disadvantages of different bone preparation techniques. The most useful bones appear to be long bones as they can be sectioned to increase surface area. The author found that soaking annular bone rings in a solvent overnight extracts much of the drug contained within the matrix and is not very demanding. To increase the extractability, bone could be crushed and soaked in a solvent. However, it may introduce health and safety issues for the analyst and is a much more demanding process (Drummer, 2008). The use of different preparation methods prevents direct correlation of data so results are best represented in a qualitative manner until standardization is achieved.

Table 2: Examination of Bone Preparation for Drug Isolation Methods

Sample Source and Bone Preparation	Drug Isolation Method	Drugs Detected	Source
Femoral Bone; 1-2mm bone rings	PSE: Methanol for 18h	Antidepressants, Antipsychotics, Benzodiazepines, Opiates	McIntyre et al., 2000
Iliac crest and vertebra; Bone slivers	PSE: Methanol for 16h	Antidepressants, Cocaine Benzodiazepines, Opiates	McGrath et al., 2009
Unspecified source; Whole bone and bone slivers	PSE: Methanol for 24h PSE: Water for 24h	Citalopram	Horak et al., 2002
Epiphyseal and diaphyseal sections of femora	PSE: Methanol for 72h	Ketamine, Diazepam Amitriptyline, Citalopram	Watterson et al., 2008 Watterson et al., 2009
Central Cavity and Arm Bones; Ground Bone			Watterson et al., 2012 Watterson et al., 2013
Central Cavity and Arm Bones; Ground Bone	MAE: PB6 for 15 and 30min	Ketamine, Pentobarbital, Diazepam, Meperidine	Desrosiers et al., 2009 Watterson et al., 2011
Unspecified source; Ground bone	Acid Digestion: 0.1 M HCl for 12h	Morphine 6-acetylmorphine	Guillot et al., 2007
Thighbone; Ground bone	Acid Digestion: 3 M HNO_3 for 24h	Opiates	Raikos et al., 2001

1.4 Solid Phase Extraction

Solid phase extraction (SPE) is a separation technique that involves a selective transfer of a material between a solid sorbent and a liquid phase. Separation is achieved through different affinities for phases based upon adsorption, size and charge. There are three main mechanisms of compound retention on a sorbent: reversed phase, normal phase and ion exchange. In reverse

phased SPE, the stationary phase is non-polar (silica or a polymer backbone, modified with alkyl or aryl groups) (Arsenault, 2012). Compound retention is a result of hydrophobic (non polar) interactions between the C-H bonds in the analyte and C-H bonds on the sorbent. In normal phase SPE, the stationary phase is polar (silica modified with alkyl chains containing polar functional groups) (Arsenault, 2012). Interactions occurring between polar functional groups of the analyte and polar functional groups of the sorbent are responsible for compound retention. Polar interactions may include hydrogen bonding, pi-pi interactions, and dipole-dipole interactions.

Ion exchange SPE is broken down into two groups: anion exchange and cation exchange. Anion exchange stationary phases are composed of either quaternary amine functional groups (strong anion) or secondary amines (weak anion) bonded to a silician backbone (Arsenault, 2012). Cation exchange stationary phases are composed of either sulfonic acid groups (strong cation) or carboxylic acid functionalities (weak cation) bonded to a silician backbone (Arsenault, 2012). Compounds are retained on the basis of the attraction between the charged functional group on the analyte and that on the sorbent. To ensure that attraction occurs, the pH of the aqueous solution must be sufficient to charge both functionalities within the analyte and on the sorbent. Mixed mode SPE sorbents make use of two mechanisms to achieve compound isolation; reversed phased (alkyl functional groups) and ion exchange (amine or acid functionalities) (Arsenault, 2012). Compounds are initially retained to the sorbent by hydrophobic interactions. Basic samples are acidified which charges the amino group and allows the sample to bind to the sorbent through ionic interactions.

SPE can be divided into five major steps: conditioning, equilibration, loading, washing and elution. Conditioning is performed, using a strong organic solvent (e.g. methanol), to wet the porous surface (activation of the stationary phase) and create a pathway between the sorbent and the sample (Arsenault, 2012). Equilibration, using water and the sample solvent, replaces the methanol in the pores with sample solvent to allow compounds of interest to interact with the sorbent (Arsenault, 2012). Samples, diluted in the same aqueous solvent as the equilibration step, are loaded onto the sorbent and allowed to flow by gravity (Arsenault, 2012). Washing, using a series of solvents, is performed to remove matrix constituents with less retention to the sorbent and may interfere with the analysis (Arsenault, 2012). Within the washing step, sorbents are

dried using a vacuum which removes any residual solvent and water within the sample. Elution is performed using a solvent that is able to break interactions between the analyte and the sorbent (Arsenault, 2012). In the case of basic compounds, a strong organic solvent with a small amount of ammonia is used to neutralize a charged amine group and allow analyte desorption into the solvent.

1.5 Gas Chromatography Mass Spectrometry

Chromatography began in the 1900s with Mikhail Tvest who examined the application of liquid samples to homemade columns of various adsorbents (Sparkman et al., 2011). In 1950, Martin and James developed gas chromatography (GC) after reading a report about partitioning chromatography, published in 1941 (cited in Sparkman et al., 2011). Mass Spectrometry (MS) began in the late 1800s with Thompson and Wien and was dominated by analyzing nuclides that made up known elements until the 20th century when petroleum products and organic compounds became the focus of MS (cited in Sparkman et al., 2011). The first reports of interfacing these two techniques were illustrated in 1956 using a GC and time of flight (TOF)-MS and in 1957 using a GC and a magnetic sector MS (Sparkman et al., 2011). At this time, data were manually entered into a computer. In 1965, the first minicomputer was developed by Digital Equipment Corporation to acquire and process GC-MS data (Sparkman et al., 2011). By the 1900s, faster data systems were developed and were being routinely used (Sparkman et al., 2011). Today's instrumentation has seen significant improvements in areas such as the injector, columns, ionization sources and flow and pressure control and the compact size allow for bench-top applications. These have all led to user-friendly instrumentation and better sensitivity.

Chromatography is an analytical technique that separates constituents in a sample based upon their interaction between the mobile and stationary phases. Separation is a result of different distribution coefficient for various constituents. The distribution coefficient is a function of the time spent in the stationary phase and time spent in the mobile phase (Skoog et al., 2010). A constituent that spends more time in the stationary phase will be retained longer. The opposite is true of a constituent that spends less time in the stationary phase. The efficiency of separation is based upon two factors: differences in elution time (function of distribution coefficient) and how broad the peaks are (diffusion) (Skoog et al., 2010). The van Deemter equation illustrates how multiple paths (A), longitudinal diffusion (B) and the mass transfer between the mobile phase

and stationary phase (C) will impact the efficiency of a separation (H; resolution). The equation is shown below:

$$H = A + \frac{B}{\mu} + C\mu \text{ (Skoog et al., 2010)}$$

where μ is the linear velocity (Skoog et al., 2010). The multiple paths term refers to how many possible paths the molecule can take through the column to reach the detector. In open tubular columns, this term goes to zero. Longitudinal diffusion refers to the extent of band broadening in the sample. The mass transfer term refers to the equilibrium of the samples between the mobile and stationary phases. For a given sample, if the travel time through the column is too long, this results in a slower interaction between the mobile and stationary phases and the peaks will broaden (short and robust) which will decrease resolution.

Plate height (H) is a measure of column efficiency. As particle size decreases, efficiency (number and height of theoretical plates) increases. The length of the column (L) will also affect efficiency as shown in the equation below:

$$N = \frac{L}{H} \text{ (Skoog et al., 2010)}$$

where N represents the number of theoretical plates. As the length of a column increases, there are more stationary phase particles which results in more theoretical plates and a decreases plate height. The number of theoretical plates is inversely proportional to the peak width (Skoog et al., 2010). Short peak widths result in tall, narrower bands, minimizing diffusion. Narrower peaks provide a greater peak capacity per unit time; increasing efficiency (Skoog et al., 2010).

In GC-MS, a liquid sample is loaded into an injection port, where it becomes vaporized, and is transported through the column, containing the stationary phase, by a gaseous mobile phase (carrier gas) to the detector (mass spectrometer). Helium is commonly used as the carrier due as opposed to hydrogen and nitrogen, to its non-explosive nature compared to hydrogen and solutes are better able to rapidly diffuse through it compared to nitrogen (Skoog et al., 2010). Open tubular columns made of fused silica are generally used as they offer higher resolution, and shorter analysis times compared to packed columns (Skoog et al., 2010). There are many factors that can affect the efficiency of a column which include stationary phase thickness, column diameter and column length. The majority of GC analyses involve temperature programming.

The temperature of the column is raised during the separation to terminate constituent interactions which increase the analyte vapour pressure, decrease the retention time of late eluting compounds and create narrower peak profiles (Skoog et al., 2010). This technique is more suited to volatile, non-thermally labile compounds which may limit its application for certain types of compounds. However, one way to make this technique more applicable would be derivatization. Derivatization is the chemical modification of a compound to improve its detectability using GC by way of increasing volatility and stability. These reactions fall into three categories: alkylation - esterification, silylation and acylation. Alkylation involves the replacement of active hydrogen by an aliphatic or aromatic group resulting in less polar compounds (Orata, 2012). In silylation, active hydrogen is replaced by a silyl group reducing the polarity of the compound (Orata, 2012). Acylation involves the replacement of active hydrogen with acyl group rendering the compound less polar (Orata, 2012).

After separation, compounds are passed through a transfer line and enter the mass spectrometer. Compounds enter the ionization chamber and undergo ionization by electron impact ionization (EI). EI is the most commonly used form of ionization and involves the interaction of compounds with a high energy electron beam, producing a positively charged molecular ion (M+). The high energy electron beam is produced by applying a set voltage, 70 eV, to a heated filament which forces the electrons to move from the surface of the filament to ground (Levine, 2010). These high energy electrons cause destabilization of the compounds which results in rearrangement and bond breakage, ultimately fragmenting the compound (Levine, 2010). EI produces unique, reproducible spectra that can be compared to standard libraries to aid in identification. Following ionization, ions are passed into the mass analyzer. The single quadrupole is a commonly used mass analyzer that is composed of four metal rods that are arranged in parallel sets to which a constant and radio-frequency voltage are applied (Levine, 2010). Adjustment of the radio-frequency voltage will permit ions, with a defined set of mass-to-charge ratios, to pass through the rods and enter the detector (Levine, 2010). Ions without the defined mass-to-charge ratios will deflect into the rods (Levine, 2010). Target analyses operate single quadrupoles in selected ion monitoring mode (SIM) due to the increased sensitivity and selectivity. This involves monitoring specific ions (usually includes one quantifier and two qualifiers) for each compound of interest (Levine, 2010). Monitored ions travel to the detector which measures and converts them to intensity, producing a mass spectrum.

1.6 Drug Detection in Human Bone and Bone Marrow

The first study to use skeletal tissues as a biological specimen was conducted by Noguchi et al. in 1978. The authors used bone marrow from the odontoid process of the top eight vertebrae. Amitriptyline (0.07 mg) was recovered from 51.8 g bone marrow sampled (Noguchi et al., 1978). From this study, the police were able to search local pharmacies and determine that the deceased had been given a prescription for amitriptyline (Noguchi et al., 1978). Since this was the first discovery of drugs in skeletal tissues, the authors could not make any definitive conclusions but this illustrated a possibility for further use of skeletal tissues. Seven years later, sternum samples, along with cadaver organs and blood, were used to detect barbiturates and glutethimide (Benko, 1985). Out of the ten cases examined, amobarbital (n=7), glutethimide (n=5), phenobarbital (n=2) and butobarbital (n=1) were found (Benko, 1985). Some other earlier studies involved the detection of methamphetamine and amphetamine thigh bone marrow following five years of decomposition in 1986 (Okamoto et al., 1986) and triazolam in bone marrow following four years of decomposition in 1997, which was used to confirm suspected murders statements (Ludo et al., 1997).

The most extensive and significant study examining the use of skeletal tissues in humans was conducted by McIntyre and colleagues in 2000. The authors examined rings of mid-femoral bone of chronic prescription drug users along with routine blood samples collected after autopsy. Decomposed bodies were excluded from analysis. Drugs were detected in bone in 25 out of the 29 blood positive cases (McIntyre et al., 2000). Diazepam (n=16) and nordiazepam (n=13) were the most commonly detected drugs (McIntyre et al., 2000). Eleven cases involved antidepressants with the most commonly detected being amitriptyline, nortriptyline, doxepin and dothiepin. Antipsychotic drugs detected include thioridazine (n=4), chlorpromazine (n=1) and clozapine (n=1). Other drugs detected include: propoxyphene, carbamazepine, methadone, codeine, oxycodone and chlormethiazole. McIntyre and colleagues also examined bone marrow from blank cases (people not using the drugs) and chronic users of prescription drugs. The first study involving bone marrow aimed at determining whether or not drugs could be detected in femoral bone marrow (McIntyre et al., 2000). Some of the drugs detected include: amitriptyline (n=2), nortriptyline (n=2), doxepin (n=4), nordoxepin (n=2), sertraline (n=1), moclobemide (n=1) and chlorpromazine (n=1). Similar to bone, benzodiazepines were detected in the most

cases; diazepam (n=5), nordiazepam (n=6), temazepam (n=3) and oxazepam (n=3). Other drugs included: propoxyphene, methadone and oxycodone.

The second study involving bone marrow aimed to determine a correlation between human blood drug concentrations and lumbar bone marrow. Drugs detected in lumbar bone marrow include mianserin, nordoxepin, doxepin, sertraline, moclobemide, thiordazine, mesoridazine and olanzapine. Similar to the femoral bone marrow study, benzodiazepines were detected more frequently than other drug classes. In all positive bone marrow cases, the bone marrow concentrations were greater than the blood concentrations (McIntyre et al., 2000). Blood and bone marrow concentrations for benzodiazepines showed a positive correlation indicating that bone marrow concentrations may be representative of post-mortem drug concentrations in blood. The varying amount of positive cases in each bone marrow study may be a result of the type of bone marrow used. Femoral bone marrow is mainly yellow whereas lumbar bone marrow is mainly red and both have different affinities for drugs. This study provided positive evidence that multiple classes of drugs can be detected in skeletal tissues and these may serve as useful alternatives to standard specimens.

Opiate concentrations were determined in fresh bone, bone marrow and buried bone of a deceased heroin addict in 2001 (Raikos et al., 2001). The fresh thighbone had a morphine concentration of 340 ng/g while the buried bone gave a morphine concentration of 155 ng/g which represented a 54.4% loss in morphine concentration (Raikos et al., 2001). One other interesting finding was that the bone weighed 17% less after burial (Raikos et al., 2001). This was the first study to directly compare drug concentrations in fresh and decomposed bone tissue and it indicates that the post-mortem environment has an influence on the observed drug concentrations. A second, larger scale, study was conducted by McGrath and Jenkins in 2009 that looked at drug detection in bone where toxicological analysis yielded a positive blood result (McGrath et al., 2009). The authors used individuals ranging from newborn to 81 and analyzed the iliac crest in adults and vertebra in infants (McGrath et al., 2009). Decomposed remains were excluded from analysis. Amitriptyline, citalopram, meperidine, cocaine, codeine, and benzodiazepines were among the most frequently detected drugs in both blood and bone. The authors noted that drugs were less likely to be detected in bone however when present, the bone usually gave higher concentrations (McGrath et al., 2009). Also, the authors saw no correlation

between blood concentrations and bone concentrations (McGrath et al., 2009). This study reiterated the potential of skeletal tissues to determine drug exposure.

1.7 Drug Detection in Animal Bone and Bone Marrow

Winek and colleagues (1982) completed a large amount of research on fresh skeletal tissues using rabbits as the model specimen. Rabbits were dosed intraperitoneally with a variety of drugs to see if there was a positive correlation between bone marrow and plasma concentrations. The lipid content of bone marrow samples was taken into account in some studies to examine if it had any effect on the observed blood to bone marrow ratios. The blood to bone marrow ratios were improved when corrected for lipid content for both methanol (Winek et al., 1982) and isopropanol (Winek et al., 1982). In the study involving isopropanol, acetone was a produced during the metabolism and its blood to bone marrow ratios were also calculated. Acetone was affected by storage conditions and delays between death and analysis hindering its correlation ability (Winek et al., 1982). All other drugs investigated showed strong, linear relationships between blood and bone marrow concentrations, except flurazepam. Overall the data implies that bone marrow concentrations can be used to predict blood concentrations. Table 3 shows the blood to bone marrow ratios and the correlation data for drugs examined by Winek and colleagues.

Gorczynski et al. (2001) examined midazolam in bone tissue and bone marrow following acute and chronic administration in mice. Midazolam was detected in both exposure patterns for the bone marrow but only in the chronic exposure for the bone. An important consideration in this study is that the authors indicated they had not removed all soft tissue from the bone samples so they could not exclude the possibility that the positive concentrations may have come from the soft tissues. This study also looked to examine the effect of freezing samples as well as burying samples in sterile and non-sterile soil, on amount of drug observed in chronic dosing. Bone marrow concentrations of midazolam were the same for both frozen samples and samples that were buried in sterile soil, but were not detectable in samples buried in non-sterile soil (Gorczynski et al., 2001). Midazolam was detectable in all situations for the bone samples with the highest concentration in the frozen samples and lowest in the non-sterile soil (Gorczynski et al., 2001). These results confirm that midazolam is in the bone itself as there is endogenous tissue present (Gorczynski et al., 2001). This study further supports the notion that

Table 3: Summary of Winek Studies illustrating the ratio between blood and bone marrow concentrations

Drug Examined	Blood to Marrow Ratio	Correlation Results	Reference
Methanol	Observed: 2.57 ± 0.61 Corrected: 1.60 ± 0.34	Positive correlation	Winek et al., 1982
Isopropanol	Observed: 2.26 ± 0.38 Corrected: 1.45 ± 0.17	Positive correlation	Winek et al., 1982
Acetone	Observed: 2.81 ± 0.71 Corrected: 1.82 ± 0.47	Positive correlation	Winek et al., 1982
Flurazepam	0.029 ± 0.012	Positive correlation $R^2 = 0.878$	Winek et al., 1982
Pentobarbital	1.06 ± 0.05	Strong, positive correlation $R^2 = 0.950$	Winek et al., 1985
Desipramine	37.204 ± 4.461	Strong, positive correlation $R^2 = 0.919$	Winek et al., 1990
Nortriptyline	28.92 ± 3.91	Strong, positive correlation $R^2 = 0.956$	Winek et al., 1993

post-mortem environment will influence observed drug concentrations. Guillot and collaborators examined morphine and 6-acetylmorphine (6-AM) concentrations in mice bone marrow and bone following acute and chronic administration of heroin (diacetylmorphine). Morphine and 6-AM were detected in blood, bone marrow and bone following the acute exposure, with morphine having higher concentrations than 6-AM, but not in the chronic case. There was no correlation between blood, bone marrow and bone the authors suggest this is a result of the given lethal dose not attaining proper equilibrium (Guillot et al., 2007). The authors also examined bones that had been stored on soil in a jar for two months. 6-AM was detectable in the bone marrow but had a

71% loss, whereas morphine underwent a 100% loss (Guillot et al., 2007). After two months of storage, no drugs were detectable in the bone samples (Guillot et al., 2007). This study showed significant variation in the concentrations observed. This was due, in part, to the mice not being sacrificed at the same time; all were sacrificed 3 to 8 minutes post-dose.

The most recent work examining drugs in skeletal tissues was conducted by Watterson and colleagues (2008). Some of the first reported work involved the detection of drugs in rat bone and bone marrow using ELISA. The ELISA data were presented in terms of a percent decrease in absorbance (%DA) to account for cross-reacting endogenous compounds and to make the ELISA data increase with increasing drug concentration (Watterson et al., 2008). Rat bone and bone marrow were assayed for ketamine following different periods of delay between drug exposure and death, using an acute dose. The authors noted that as the dose-death interval increased, the %DA decreased and there was no significant difference between the %DA for the 15 and 30 minute dose-death intervals (Watterson et al., 2008). The authors also examined the %DA values in different sections within a bone and found that epiphyseal sections (distal ends) had a greater ketamine %DA than the diaphyseal sections (midsection) of the femurs. Also, epiphyseal sections showed a difference in response for the groups sacrificed after 15 and 30 minutes but no difference was displayed in diaphyseal sections (Watterson et al., 2008). A similar study was conducted using diazepam. The dose-death intervals varied significantly in the bone marrow samples and the epiphyseal sections but not in the diaphyseal sections (Watterson et al., 2008).

Watterson and colleagues also completed a study involving examining the distribution of drugs in decomposed skeletal tissues. Amitriptyline, pentobarbital and diazepam were examined in twelve different bones types of a decomposed pig. Drug levels are reported as mass normalized response ratios (RR/m). The largest RR/m values were observed in the rib for all bone types examined (Watterson et al., 2010). Large RR/m values were also found in the vertebra, pelvi and femora (Watterson et al., 2010). ANOVA analysis was performed and showed bone type to be a main effect with respect to the drug levels observed. The authors also noted that examination of ratio of levels of diazepam and nordiazepam showed less variability than individual drug RR/m values (Watterson et al., 2010). As a result, the authors completed a similar study, using the same pig, to determine if the trend of lower site-dependent variability in the ratio of parent drug and

metabolite extended to amitriptyline and citalopram using thirteen different bone types. Once again, ANOVA showed bone type to be a main effect for the drug levels observed (Desrosiers et al., 2012). It was found that the mean levels (RR/m) of individual drugs varied by a factor of 45 and 33 for amitriptyline and nortriptyline across all bone types examined while citalopram and desmethylcitalopram varied by a factor of 166 and 95 respectively (Desrosiers et al., 2012).The mean levels (RR/m) of the ratio of parent drug and metabolite showed much less variability, varying only 1 to 8-fold across all bone types examined (Desrosiers et al., 2012). The relative stability of the ratio of levels of parent drug and metabolite compared to the levels of the individual drugs may provide valuable insight into accessing the circumstances of drug exposure (Desrosiers et al., 2012).

Their most recent work involves discriminating different patterns of drug exposure by way of levels of individual drugs as well as the ratio of levels of parent drug and metabolite. Ketamine and norketamine were examined in multiple bone tissues following acute and repeated exposures (Watterson et al., 2012). This study found that ketamine drug levels were able to discriminate patterns of drug exposure for all bone types while norketamine and the ratio of levels of ketamine and norketamine were able to discriminate patterns of drug exposure for the majority of bone types examined (Watterson et al., 2012). Pooled levels were also able to discriminate drug exposure patterns. Amitriptyline, citalopram and metabolites were also examined in a similar study (Watterson et al., 2013). Individual drug levels (RR/m) of AMI, CIT and their metabolites did not differ significantly between the two exposure types for individual bone types, in most cases, or for the pooled comparison of all bone types, in all cases (Watterson et al., 2013). Conversely, the ratio of levels of metabolite and parent drug ($RR_{metabolite}/RR_{parent}$) differed significantly within each bone type between acute and repeated exposures in most cases. In the analysis of pooled bone data, levels of $RR_{metabolite}/RR_{parent}$ differed significantly between exposure patterns in all cases. For analysis of citalopram and metabolites, values of RR_{DDMCIT}/RR_{DMCIT} showed the greatest discrimination between exposure patterns (Watterson et al., 2013). This result indicates that is important to consider more than just the primary metabolite and its relationship to the parent drug (Watterson et al., 2013). These studies indicate that when using skeletal tissues, the relationship between the parent drug and metabolite may provide more insight into the drug exposure pattern.

1.8 Pharmacology of Ketamine

Ketamine (KET), (RS)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone, is a basic drug with a pKa of 7.5 and an analog of phencyclidine (PCP) (Levine, 2010). Usually found as a hydrochloride salt under the generic name Ketalar, it is used as an anesthetic and/or analgesic in human and veterinary medicine (Katzung et al., 2009). KET has become an abused drug due to the production of an anesthetic state termed dissociative anaesthesia. This is characterized by catatonia, amnesia and analgesia with or without the loss of consciousness (Kohrs et al., 1998). KET acts as an antagonist on N-methyl-D-aspartate (NMDA) receptors and inhibits glutamate activation of the channel in a non-competitive manner (Kohrs et al., 1998). The blockage is time-, concentration- and frequency-dependent (Korhs et al, 1998). This is considered to be the primary site of KET's anesthetic action (Korhs et al., 1998). KET displays agonist activity on the κ-opioid receptor, which induce psycho-mimetic reactions typical in KET anesthesia and has some antagonist activity on the μ-receptor (Korhs et al., 1998). KET has also been shown to interact with other receptors including muscarinic acetylcholine receptors, sodium channels and L-type calcium channels. A summary of the effects produced by each receptor type are found in Table 4. Providing an individual with a dose of diazepam, midazolam or propofol prior to KET will reduce some of these adverse effects (Katzung et al, 2009). KET in conjunction with other intravenous and inhaled anesthetics has been used as an alternative to opioid analgesics for ventilator depression (Katzung et al., 2009).

KET is a chiral molecule that is composed of two optical isomers: 50% R (-) and 50% S (+) Ketamine (Levine, 2010).The enantiomers have a varying affinity for different receptors. S (+) KET has a fourfold greater affinity for the NMDA receptor and a twofold greater affinity for the muscarinic receptors (Korhs et al., 1998). KET is most commonly administered intravenously but has also been administered subcutaneously, intramuscularly, intranasal or orally (Levine, 2010). It has a volume of distribution of 3-4 L/kg and a half-life of 3-4 hours in humans (Levine, 2010). The half-life of KET in rats has been shown to range from 0.7 to 2.5 hours (Williams et al., 2004). KET undergoes an N-demethylation (CYP P450 enzymes) to form its major metabolite norketamine (NKET) (Levine, 2010). NKET is thought to have one third the

Table 4: Receptor Mediated Clinical Effects of Ketamine

Receptor	Clinical Effects	References
N-methyl-D-aspartate	Hallucinations, nausea, vomiting, blurred vision, increased or decreased respiration, heart rate and/or blood pressure	(Levine, 2010)
Opioid (μ and κ)	Seizures, paranoia, delirium, psychosis, dizziness, anxiety, palpitations	(Korhs et al., 1998)
Muscarinic Acetylcholine	Memory loss, consciousness, mydriasis, amnesia, bronchial and mucus secretion	(Durieux, 1995)
Sodium Channels	Sympathetic, sensory and motor blockages	(Durrani et al., 1989)
L-type Calcium Channels	Vasodilation, bronchodilation,	(Sinner et al., 2008)

anesthetic activity of KET (White et al., 1982). NKET becomes dehydrogenated to form dehydronorketamine (DHNK) (Levine, 2010). KET and its metabolites may also undergo hydroxylation and conjugation (Levine, 2010). The majority of KET and its metabolites are excreted in the urine with 4% being unchanged drug and around 80% being conjugates (White et al., 1982). Fecal excretions account for less than less than 5% of a dose (White et al., 1982).

Analgesia begins at plasma concentrations of 100 ng/mL (Sinner et al., 2008). Anesthetic blood concentrations range from 2000 to 3000 ng/mL and individuals begin to awake between 500 and 1000 ng/mL (Sinner et al., 2008). Blood concentrations in an overdose were observed to be greater than 3000 ng/mL (Levine, 2010). KET and its metabolites have been analyzed by multiple methods. The majority of the methods used for analysis are gas chromatography with electron impact ionization mass spectrometry. Table 5 lists some of the methods used in the analysis of ketamine and its metabolites. Hijazi et al. examined the stability of ketamine, norketamine and dehydronorketamine in human serum and plasma. The human serum stability

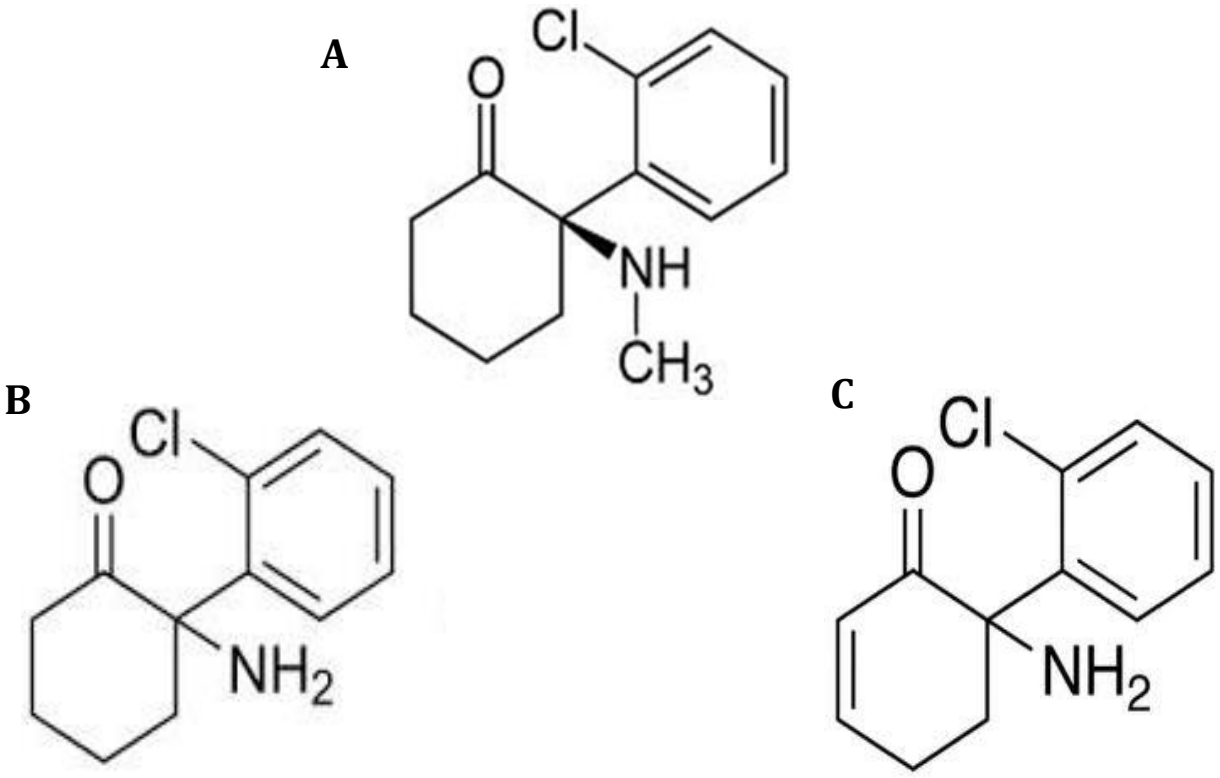

Figure 1: Structures of (A) Ketamine, (B) Norketamine and (C) Dehydronorketamine.

studies were conducted at -20˚C for 6 months and 4˚C for 2 days. The authors saw no significant difference in drug levels following storage in either condition (Hijazi et al., 2001). Human plasma stability studies were also conducted at temperatures of 4˚C and 20 ˚C for 2 hours. KET and NKET plasma concentrations were stable for 2 hours at both temperatures and DHNK plasma concentration was stable for 2 hours at 20˚C (Hijaki et al., 2001). At 4 ˚C, DHNK had a 68% loss in plasma concentration after 30min of storage (Hijaki et al., 2001). The authors believe this is a result of permeation of DHNK into the blood cells (Hijaki et al., 2001). The authors concluded that plasma at room temperature should be analyzed immediately to avoid any decrease in DHNK concentrations.

Table 5: Methods used to detect Ketamine and its Metabolites in Biological Specimens

Analytical Method and Sample Type	Sample Preparation	LoD	Reference
GC-FID; plasma	Liquid liquid extraction	KET: 500 ng/mL	(Hodshon et al., 1972)
GC-NPD; urine	Hexane extraction	KET: 5.0 ng/mL NKET: 5.6 ng/mL	(Lin et al., 2005)
GC-EI-MS; fingernails	Hydrolysis, ethyl acetate extraction	KET: 0.094 ng/mg NKET: 0.094 ng/mg	(Kim et al., 2010)
GC-EI-MS; hair	Digestion, liquid liquid extraction	KET : 0.05 ng/mg NKET : 0.05 ng/mg	(Xiang et al., 2006)
GC-EI-MS; urine	Ethyl acetate extraction, derivatization	KET: 2 ng/mL NKET: 1 ng/mL DHNK: 6 ng/mL	(Lin et al., 2006)
GC-EI-MS; urine	Solid phase extraction, derivatization	KET: 0.5 ng/mL NKET: 0.5 ng/mL DHNK: 0.5 ng/mL	(Huang et al., 2005)
GC-PICI-MS; urine	Solid phase extraction	KET: 25 ng/mL NKET: 25 ng/mL	(Kim et al., 2008)
HPLC-PDA; plasma	Liquid liquid extraction	KET: 5 ng/mL NKET: 5 ng/mL DHNK: 10 ng/mL	(Bolze et al., 1998)
LC-ESI-MS; urine	Solid phase extraction	KET: 0.03 ng/mL NKET: 0.05 ng/mL	(Parkin et al., 2008)
LC-APCI-MS; plasma	Dilution with water	KET: 0.95 ng/mL NKET: 0.48 ng/mL DHNK: 0.33 ng/mL	(Chen et al., 2007)

Chapter 2

2 Microwave Assisted Extraction of Ketamine and its Metabolites in Skeletal Tissues

The use of Microwave Assisted Extraction (MAE) to extract ketamine and its metabolites from decomposed bone tissue is described. Wistar rats received 75 mg/kg (i.p.) ketamine (n=12) or remained drug-free (controls, n=4). Following euthanasia, rats were allowed to decompose to skeleton outdoors. Bones (vertebrae, rib, pelvis, femora, tibiae¸ humeri and scapulae) were collected and pooled. Analyte stability to MAE in methanol or acetone: hexane mixture (1:1, v/v) in a closed vessel microwave system was assessed, and acetone:hexane was chosen as the MAE solvent. Bone samples underwent MAE, and extracts were assayed using microplate solid phase extraction (MPSPE) in a mixed-mode configuration and gas chromatography-mass spectrometry. Drug levels, expressed as mass normalized response ratios, were used to characterize extraction time and the effect of MAE solvent volume on the extraction time and relative recovery for each analyte and subsequent comparison to assays of extracts obtained through passive solvent extraction (PSE). Extraction time varied slightly with solvent volume, while relative analyte recovery did not differ significantly. Recovery was at least 90% of maximal value within the first 5 min for all samples examined and maximum recovery occurred within 20 minutes for MAE and 3 hours for PSE. MAE in combination with MPSPE offers a substantial improvement in analytical throughput for toxicological analysis of skeletal remains.

2.1 Introduction

Over the last fifteen years, there has been an increasing amount of research involving the toxicological analysis of skeletal tissues (McIntyre et al., 2000, Gorczynski et al., 2001, Horak et al., 2005, McGrath et al., 2009, Desrosiers et al., 2012, Watterson et al, 2012). The majority such analyses reported have used passive methanolic extraction to isolate the drugs from bone samples (Horak et al., 2005, McGrath et al., 2009, Watterson et al, 2009, 2010). These methods reported very time consuming extractions, utilising 12-72 hours of incubation time (McIntyre et al., 2000, Gorczynski et al., 2001, Horak et al., 2005, McGrath et al., 2009 Desrosiers et al., 2012, Watterson et al, 2012). Given the wide variability in detected drug and metabolite levels

across different bone types (Watterson et al., 2010, 2011, Desrosiers et al., 2012, Watterson et al., 2012), new advances in this area will require significant increases in the number of samples analysed, which, in turn, underscores the need for a method with a higher analytical throughput. One such approach would be the use of Microwave-Assisted Extraction (MAE). MAE has been used to detect compounds in environmental samples (soil and sediment) including polycyclic aromatic hydrocarbons and polychlorinated biphenyls (Bartolomé et al., 2005), endocrine disrupting chemicals (Liu et al., 2004) and triazines (Xiong et al., 1999). MAE has also been used to detect drugs in biological matrices including cocaine and opiates (Fernandéz et al., 2007, 2009), tricyclic antidepressants (Wózniakiewicz et al., 2008) and anti-inflammatory drugs (Fernandéz et al., 2013).

In MAE, microwave radiation is absorbed by the solvent and/or sample, which leads to sample heating by two mechanisms: ionic conduction and/or dipole rotation. Ionic conduction is a result of resistive heating of the solution as ions migrate under a changing electric field. Dipole rotation involves rearrangement of the molecular dipoles under the influence of the oscillating electric field. Molecular dipoles are unable to realign with the oscillating field, and undergo vibrations that create frictional heating. This process allows the heating to be applied in a focused manner towards the matrix of interest which results in desorption of analytes and dissolution in the solvent (Letellier et al., 1999, Mandel et al., 2007).The ability of a material to absorb microwaves is a function of its dielectric loss factor, which is related to the efficiency of converting microwave energy into heat and is a function of the permittivity of the material. Polar solvents, such as methanol and ethanol, have higher dielectric constants than non-polar solvents so they will absorb microwave energy with a greater efficiency. In MAE, the microwave irradiation directly heats the absorbing material, with minimal reliance on conductive and convective heat transfer. Factors that have been shown to influence microwave assisted extraction include solvent polarity, volume, time, moisture, power and temperature (Letellier et al., 1999, Mandel et al., 2007).

This laboratory has conducted various studies of the disposition of ketamine (KET) and its major metabolite norketamine (NKET) in skeletal tissues (Watterson et al., 2008, 2011, 2012). However, none of this work has examined dehydronorketamine (DHNK), a secondary metabolite of ketamine. The overall purpose of this work was to improve our sample preparation

protocols for analysis of ketamine and its metabolites, including validation of the analysis of DHNK as well as KET and NKET, in bone tissue by moving to a microwell plate-based solid-phase extraction assay, with incorporation of MAE as the means to isolate the analytes from solid bone. Improved throughput in sample preparation is critical to expanding the catalogue of data describing drug and metabolite disposition in skeletal tissues after various patterns of exposure, which is vital to improving our ability to derive toxicological information from these measurements. This study reports the effect of solvent choice, solvent volume and irradiation time for the recovery of KET and its metabolites (NKET and DHNK). The drugs of interest were extracted from pooled, ground bone tissue from acutely exposed animals that underwent outdoor decomposition for 3 weeks. Tissue extracts were analyzed by gas chromatography mass spectrometry (GC-MS).

2.2 Experimental

2.2.1 Chemicals

Methanol used in the drug extraction was HPLC grade and obtained from EMD Chemicals (Gibbstown, NJ). KET and NKET (Cerillant, Round Rock, Texas) were obtained as 1.0 mg/mL methanolic solutions and diluted as required. KET-d4 and NKET-d4 (Cerillant, Round Rock, Texas) were obtained as 100 µg/mL methanolic solutions and diluted as required. DHNK (Cerillant, Round Rock, Texas) was obtained as a 100 µg/mL acetonitrile solution and diluted as required. Acetonitrile used was reagent grade and obtained from J.T. Baker (Center Valley, PA). Phosphate buffer (PB6) was prepared by dissolving 13.8 g of monobasic sodium phosphate in 1 litre of water. This solution was adjusted to pH 6.0 using 4M sodium hydroxide. All other chemicals were reagent grade and obtained from EMD chemicals.

2.2.2 Animals and Drug Administration

Male Wistar rats (n=12) acutely exposed to ketamine were donated by Dr. T.C. Tai of the Northern Ontario School of Medicine. The animals used were housed at the Laurentian University Animal Care Facility. Upon arrival at the facility, the animals were given 7 days to acclimatize to their conditions. Adult male Wistar rats (Charles River Laboratories, St. Constant, QC, Canada) were housed in groups of three with Harlan Teklad ¼" bedding (Indianapolis, IN) on a 12 h light/dark cycle at a room temperature of ca. 20^{o}C. They were supplied with free

choice water and Harlan Teklad Laboratory Diet 8640. The procedures used in this experiment were approved by the Laurentian University Animal Care Committee. The rats were given 75 mg/kg KET (i.p.) and euthanized by CO_2 asphyxiation within 20 minutes of drug administration. This dose was chosen as it is an anesthetic dose in rats. The rats were placed in cages outdoors at a rural Ontario site and allowed to decompose for 3 weeks during late spring (May-June) with full exposure to weather features (sunlight, precipitation etc.). Drug free rats (n=4) were placed outdoors and exposed to the same conditions and serve as negative controls. Bones collected for analysis include vertebrae, ribs, pelvis, tibiae, femora, scapulae, radii, ulnae and humeri.

2.2.3 Bone Preparation

All bone types collected for rats undergoing the same drug exposure were pooled for analysis (i.e., drug positive vs. drug free). All bones were washed sequentially with phosphate buffer (PB6: 0.1M, pH 6.0), methanol and acetone to remove any loosely bound soil and other contaminants. Bones were left out to air-dry for 48 hours. Bones were first ground using a Micro-Mill grinding mill (Bel-Air Products, Pequannock, NJ) for 2min followed by pulverization to a fine powder using a Ball Mill (SPEX Sample Prep., Metuchen, NJ) for 1min.

2.2.4 Solid-Phase Extraction and Gas Chromatography Mass Spectrometry

Samples of drug-free decomposed bone (100g) were sonicated in 500 mL PB6, to prepare the drug free bone tissue extract (BTE) used in method validation. All calibrant samples were prepared in 1 mL BTE at concentrations varying from 0 to 2000 ng/mL in replicate analysis (n=3). Internal standards [200ng KET-d4 and 200ng NKET-d4] were added to each sample along with 100µL glacial acetic acid and 3 mL acetonitrile: methanol (1:1 v/v), followed by storage at -20˚C for 12h to precipitate proteins and lipids. The samples were centrifuged (1100×g) for 10 min and the supernatant was collected and evaporated to 1mL under a gentle stream of air (70˚C). The samples were diluted to 4 mL with PB6 (0.1M, pH 6.0) and acidified with 100µL glacial acetic acid.

Solid phase extraction was performed using CleanScreen XCEL 1 48 Well Plates (130 mg, United Chemical Technologies, Bristol, PA). Wells were first conditioned with 3mL methanol, 3 mL distilled water and 3 mL PB6. Samples were loaded into the wells and allowed to flow by gravity. The wells were washed with 3 mL PB6 (0.1M, pH 6.0) and 3 mL 0.1 M acetic acid, and

then dried under vacuum for 5 min at 400mmHg. After drying, wells were rinsed with 3 mL methanol. The wells were dried under vacuum for 10 min at 400mmHg. Basic analytes were eluted with 4mL 80:17:3 ethyl acetate/isopropanol/ammonium hydroxide. Extracts were then evaporated to dryness under a gentle stream of air (70°C) and reconstituted in 100 µL ethyl acetate for analysis by GC-MS.

Extracts were analyzed on PerkinElmer Clarus 600C GC–MS (PerkinElmer LAS, Shelton, CT) using electron impact ionization, in the selected ion monitoring (SIM) mode. Separation was achieved using a ZB-Drug-1 column (30 m × 0.25 mm × 0.25 µm, Phenomenex, Torrance, CA). Extracts (2 µL) were injected into the injector, which was maintained at 250°C. The GC oven temperature was initially set to 60°C and held constant for 3 min. The set temperature was directly increased to 170°C at a rate of 130°C/min and then increased to 205°C at a rate of 2°C/min. The oven temperature was then set to directly increase to 300°C and held at that temperature for 3 min at the end of the analytical run. The total GC–MS run time was 26 min. The ions monitored were *m/z* 180, 182, and 209 for KET (underlined ion used for quantitative analysis), *m/z* 166, 168, and 195 for NKET, *m/z* 153, 138 and 221 for DHNK, *m/z* 170, 172 and 199 for NKET-d4 and *m/z* 184, 213, and 156 for KET-d4. The response ratio (RR) is defined as the ratio of peak area for the quantitation ion (*m/z* 180, 166 or 153) to that of the ion with *m/z* 184 or 170 corresponding to KET-d4 or NKET-d4, respectively. NKET-d4 was used as the internal standard for DHNK.

The limit of detection and quantification were 10 ng/mL KET and NKET and 25 ng/mL DHNK. Precision (%coefficient of variation, %CV of the ratio of measured responses of m/z 180(KET), 166(NKET), DHNK (153), KET-d4 (184) and NKET-d4 (170) respectively) ranged from 2 to 19%. This assay was shown to be linear for KET (R^2 = 0.9978-0.9986), NKET (R^2 = 0.9963-0.9994) and DHNK (R^2 = 0.9966-0.9990) for all standard curves prepared.

2.2.5 MAE – Examination of Analyte Stability to Microwave Irradiation

The solvents examined in the extraction of each drug were methanol (MeOH) and acetone: hexane (ACE: HEX, 1:1 v/v). The stability of each drug was examined under microwave irradiation in the presence of each solvent system. A solution of KET, NKET and DHNK (100 and 1000 ng/mL) was prepared in each solvent (10 mL) and transferred to PTFE-lined extraction

vessels that contain 0.5g drug-free control bone. A magnetic stir bar was used in each extraction vessel to ensure sample agitation throughout the extraction. Each solvent and concentration combination was performed in triplicate with one solvent control. Extractions were performed by MARS 6 (CEM Corp., USA) at 80˚C (1200 W). For each sample, at a defined interval (0, 30 or 60 min), 1 mL of solvent was removed. Prior to solvent removal, the vessels were cooled for 20 min. After solvent recovery, each sample was evaporated to dryness to under a gentle stream of air at 70˚C. The samples were reconstituted in 1 mL PB6 (0.1M, pH 6.0), extracted by the MPSPE method described above and analyzed by GC-MS.

2.2.6 MAE - Extraction Time Characterization

Bone samples (0.5g) were transferred into PTFE-lined extraction vessels and 5 mL of extraction solvent (ACE:HEX, 1: 1 v/v) was added. A stirring bar was used in each extraction vessel to ensure constant sample agitation throughout the extraction. Analysis was performed in triplicate with one negative control. Extractions were performed by MARS 6 (CEM Corp., USA) at 80˚C. For each sample, at a defined interval (5, 10, 15, 20, 30, 45, or 60 min), the solvent was recovered and replaced with clean solvent in order to monitor the time required to achieve maximum extraction yield. Prior to solvent removal, the vessels were cooled for 20 min. After each solvent recovery, samples were washed once with 5 mL of extraction solvent and pooled with the original fraction. After sample recovery, all samples were evaporated to dryness under a gentle stream of air at 70˚C. The samples were reconstituted in 1 mL PB6 (0.1M, pH 6.0), extracted by the MPSPE method described above and analyzed by GC-MS.

2.2.7 MAE - Effect of Extraction Solvent Volume on Extraction Yield

Bone samples (0.5g) were transferred into PTFE-lined extraction vessels and 5 mL (n=3) or 10 mL (n=3) of ACE:HEX (1:1 v/v) was added to each tube. A stirring bar was used in each extraction vessel to ensure constant sample agitation throughout the extraction. For each volume, triplicate analysis was performed along with one negative control. Extraction was performed by MARS 6 (CEM Corp., USA) at 80˚C. For each sample, at defined intervals (5, 10, 15, 20, 30, 45 or 60 min), the solvent was recovered and replaced with clean solvent in order to monitor the rate of extraction After each solvent recovery, samples were washed once with 5 mL of ACE:HEX (1:1 v/v) and pooled with the original fraction. After sample recovery, all samples were evaporated to dryness under a gentle stream of air at 70˚C. The samples were reconstituted in 1

mL PB6 (0.1M, pH 6.0), extracted by the MPSPE method described above and analyzed by GC-MS.

2.2.8 Passive Solvent Extraction

Bone samples (0.5 g) were weighed into screw-cap test tubes and the appropriate amount of ACE:HEX (1:1, v/v) or methanol, 5 or 10 mL, was added. For each volume, triplicate analysis was performed with one negative control sample. Extractions were performed using a hot plate at 50˚C. Each sample underwent 180 min passive extraction, with frequent vortexing to aid in analyte diffusion into bulk solvent. After sample recovery, all samples were evaporated to dryness under a gentle stream of air at 70˚C. The samples were reconstituted in 1 mL PB6 (0.1M, pH 6.0), extracted by the MPSPE method described above and analyzed by GC-MS.

2.2.9 PSE - Bone Particle Size Comparison

Two grinding systems were evaluated in terms of their extraction yield: Kitchen Aid coffee grinder and laboratory grade micromill and ball mill grinding system. Diagrams of bone particles from each grinding system can be viewed in appendix A. Bone samples (0.5g) were weighted into screw cap test tubes and the appropriate amount of methanol, 5 or 10 mL, was added. Analysis was performed in triplicate with one negative control for bones prepared with each grinding system. Extractions were performed on a hotplate set at 50˚C for 3 hours. Samples were frequently vortexed to aid in analyte diffusion. Following the extraction period, solvent was recovered and samples were washed with 5 mL methanol and the two fractions were pooled. After sample recovery, all samples were evaporated to dryness under a gentle stream of ait at 70 ˚C, reconstituted in 1 mL PB6 (0.1M, pH 6.0) and underwent solid phase extraction as described above.

2.3 Results and Discussion

2.3.1 Expression of Drug Levels

In this work, we report the drug levels as the mass normalized response ratios (RR/m) for KET, NKET and DHNK measurements, as we have done in various other reports (Watterson et al., 2008, Watterson et al., 2011, Watterson et al., 2012). This is done because analyte recovery from solid bone tissue cannot be accurately measured using conventional techniques (i.e. GC-MS or

UHPLC) because solid bone sample is heterogeneous and cannot be homogenously mixed with a drug standard. The RR/m value is directly proportional to concentration and allows the comparison of drug levels between different experimental treatments. These values can be converted to estimates of concentrations however they should only be used as approximations due to the reasons stated above.

2.3.2 Solid Phase Extraction Configuration and Chromatographic Conditions

As part of our efforts to improve throughput in our laboratory, different configurations of SPE columns (with the same stationary phase) were evaluated in terms of precision, linearity, LoD and LoQ. For both the 16 column block manifold and the 48-well microplate, samples were prepared in 1 mL BTE at concentrations varying from 0 to 2000 ng/mL in replicate (n=3). Each configuration gave similar precision and linearity and the same LoD and LoQ. Due to the increase in throughput, 48-well microplate plates were used for all MAE experiments. Multiple gas chromatography conditions were evaluated to obtain optimal separation. The initial temperature of the second ramp was varied from 170-200˚C and the ramp rate was varied from 2-6 deg/min. Based upon a standard mixture of the drugs in question, the ramps that had an initial temperature of 170˚C gave the greatest degree of resolution and had the best precision. These ramps were further tested with samples prepared in bone tissue extract at 3 concentrations (50, 500, 2000 ng/mL). Ultimately, the ramp with a rate of 2 deg/min gave the best degree of resolution and the best precision. Figure 2 shows the degree of resolution obtained with the optimized parameters.

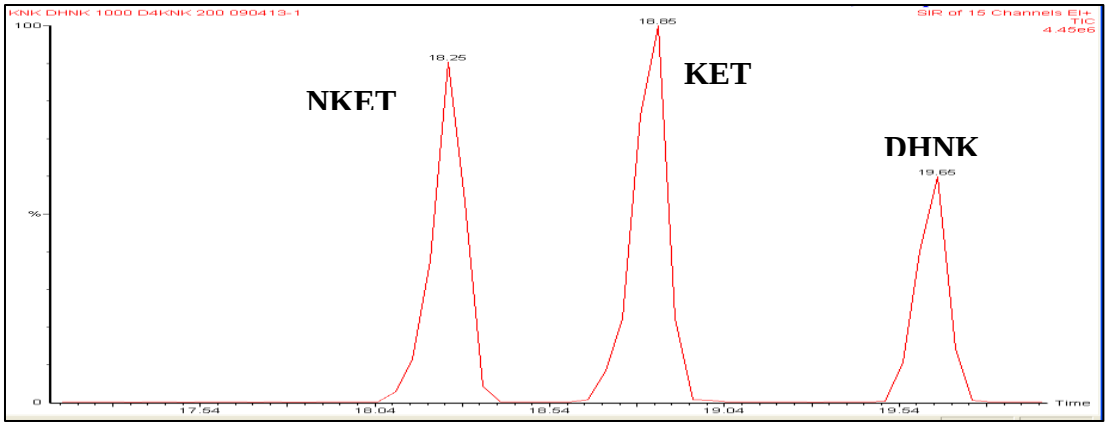

Figure 2: Gas chromatography mass spectrometry total ion chromatogram of analytes in a standard solution. Labelled peaks correspond to ketamine (KET), norketamine (NKET) and dehydronorketamine (DHNK).

2.3.3 Drug Stability under Microwave Irradiation

The effect of microwave irradiation on each drug was investigated for each solvent. Figures 3 and 4 summarize these results. Stability was assessed by preparing samples (n=3) of drug free bone in 10 mL extraction solvent spiked with each analyte at either 100 ng/mL or 1000 ng/mL. An aliquot (1 mL) of each sample was assayed before irradiation (t = 0 min), and after different periods of irradiation (30 or 60 min). In order to conclude that a given analyte was stable, the measured response ratio (RR±20%) was required to agree with RR (±20%) of the sample prior to irradiation (i.e., 0 min). For the drug standards prepared in methanol, the RR fell below the acceptable range after the 30 min time interval for samples prepared at 100 ng/mL and after 60 min for the samples prepared at 1000 ng/mL for KET, NKET and DHNK. For the drug standards prepared in acetone: hexane mixture, no measured RR fell below the acceptable range, but a substantial increase in RR was observed in all samples after the first irradiation interval, and then fell back to within the acceptable range of values after 60 min irradiation. Overall, there was more evidence of analyte stability to microwave irradiation in the presence of acetone:hexane (1:1 v/v) than in the presence of methanol. Consequently, the extraction time and solvent volume required were subsequently optimized using acetone:hexane (1:1 v/v) as extraction solvent.

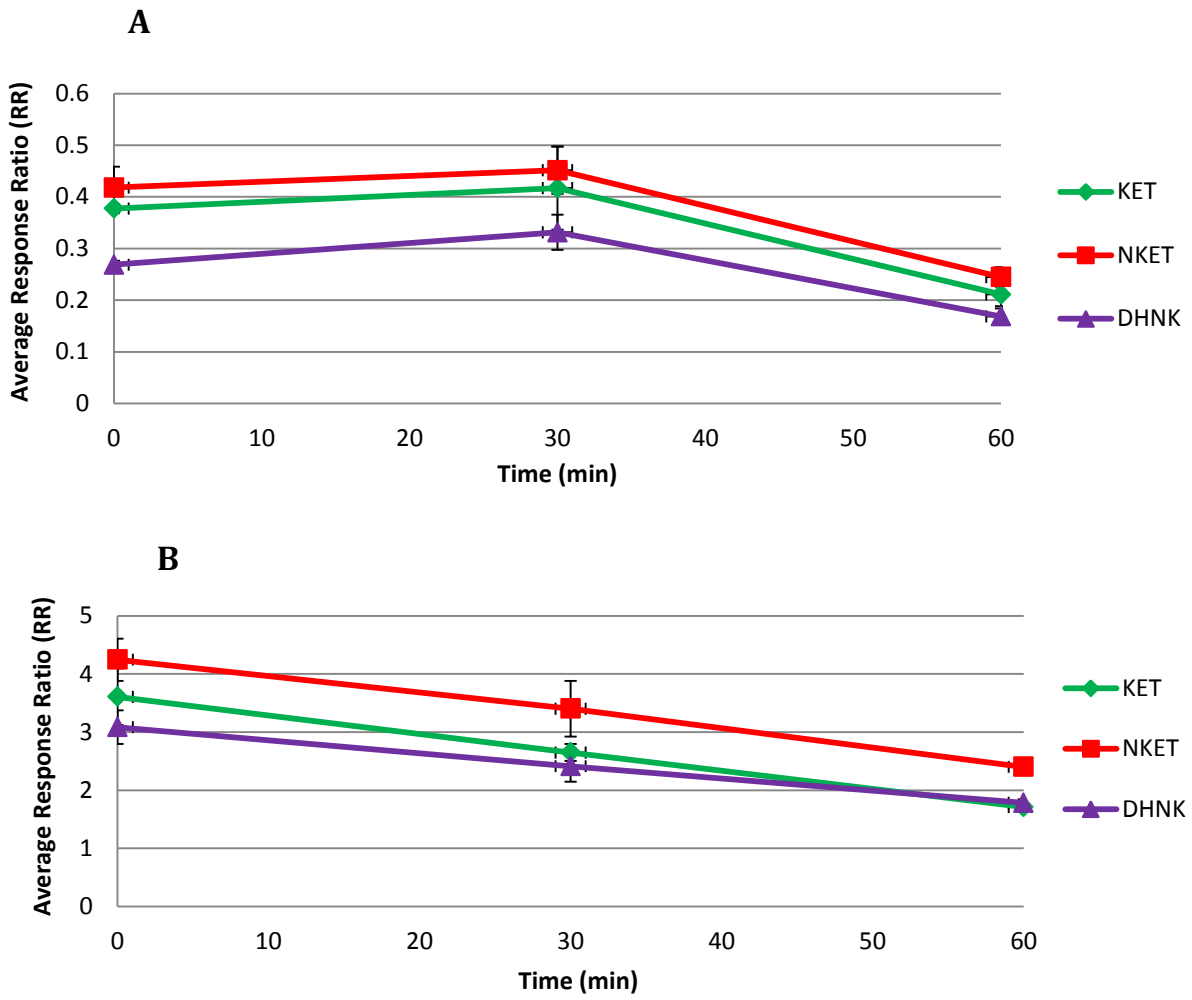

Figure 3: Stability of ketamine, norketamine and dehydronorketamine in a methanol solution prepared at (A) 100 ng/mL and (B) 1000 ng/mL following exposure to microwave irradiation. Error bars represent standard deviation.

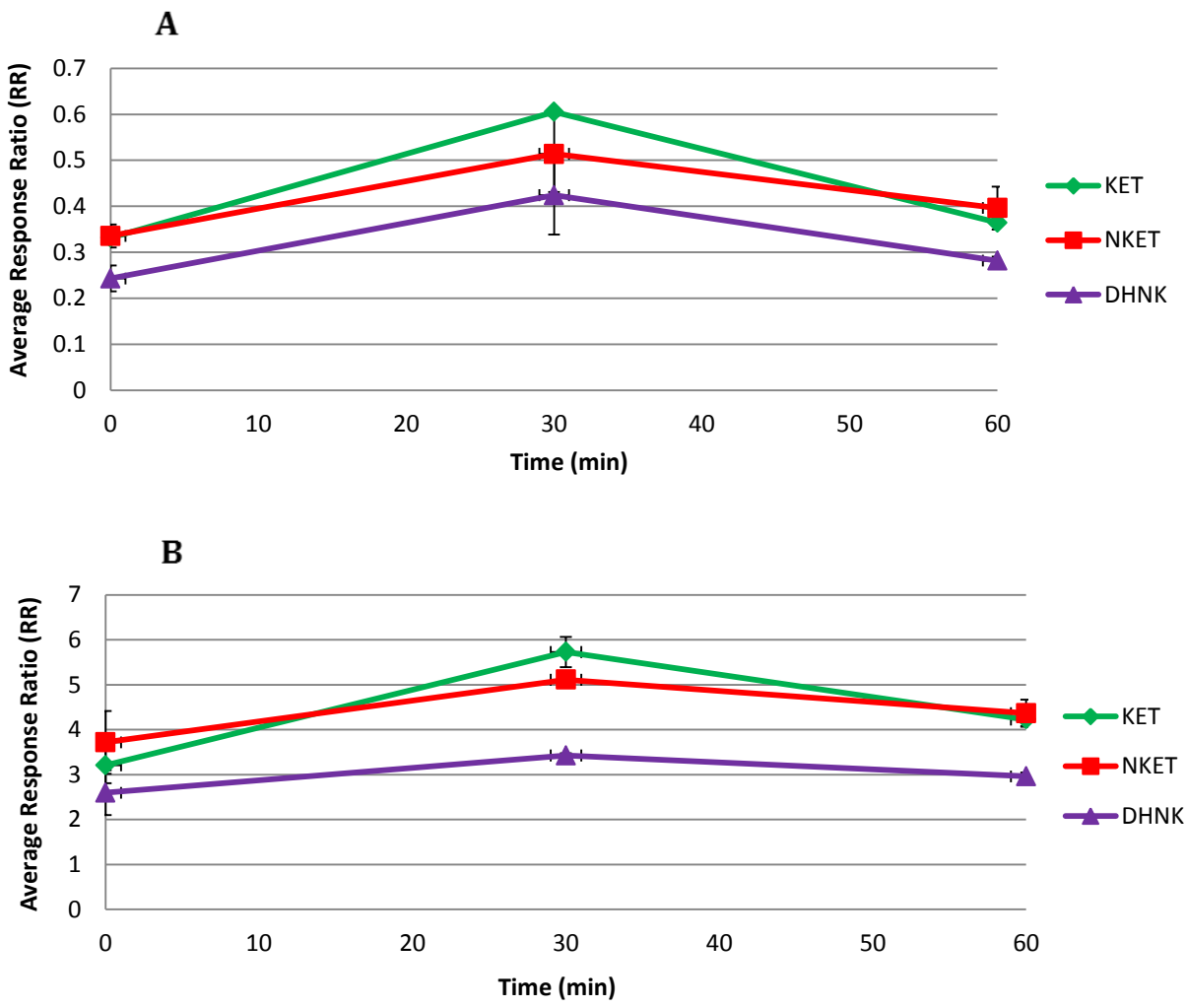

Figure 4: Stability of ketamine, norketamine and dehydronorketamine in an acetone: hexane (1:1, v/v) solution at (A) 100 ng/mL and (B) 1000 ng/mL following microwave irradiation. Error bars represent standard deviation.

2.3.4 MAE - Extraction Time

The time dependence of analyte extraction in ACE:HEX (1:1 v/v) was examined to determine the irradiation time required for routine extractions. Samples (0.5g) of drug-positive (n =3) or drug-free bone (n = 4) were added to 5 mL ACE:HEX (1:1 v/v) in PTFE extraction vessels containing a magnetic stir bar. Samples were irradiated in short intervals, and after each interval all extraction solvent was removed, the bone washed with clean solvent that was then combined with the extraction solvent from that interval. Fresh solvent was then added to each bone sample and the next irradiation interval commenced. Extraction intervals of 5, 10, 15, 20, 30, 45 and 60 min were examined. The solvent and wash samples recovered from each interval were then

assayed by the MPSPE-GC/MS method. KET and NKET were detected in these bones samples while DHNK was not. The results are summarized in Figure 5. The data in figure 4 shows that the majority of each analyte was recovered in the first 5 min of extraction. For KET, the entire extraction took 20 min to reach maximum extraction while NKET took 10 min to reach maximum extraction.

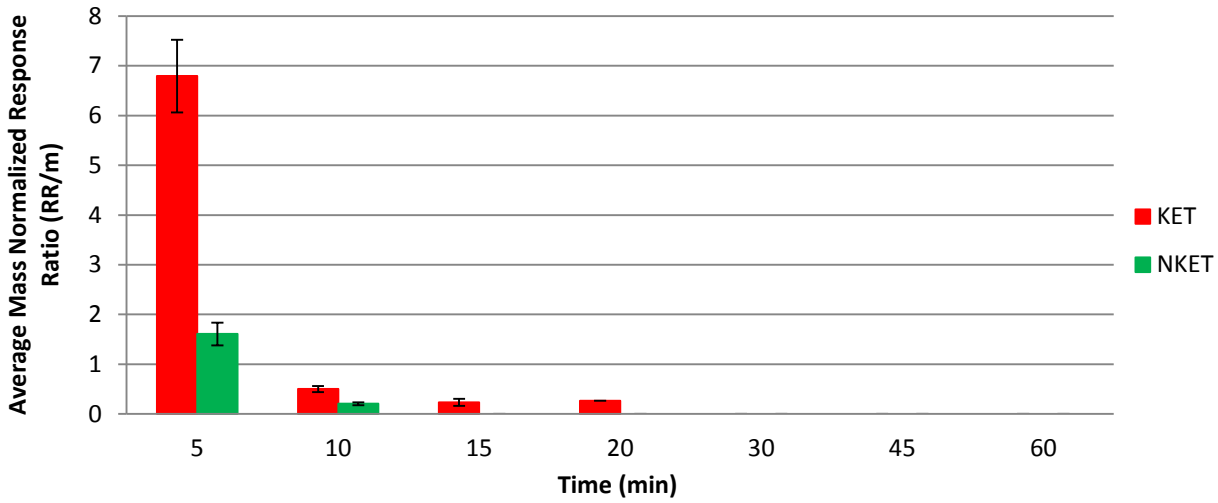

Figure 5: Incremental recovery of ketamine (KET) and norketamine (NKET) from 0.5 g bone, in acetone-hexane (1:1, v/v). Recovery is expressed as the mass-normalized response ratio (RR/m), which is directly proportional to analyte concentration. Error bars represent standard deviation.

2.3.5 MAE - Effect of Solvent Volume

Two different solvent volumes (5 and 10mL) of ACE:HEX were examined to investigate their influence on the time required for extraction and relative recovery for each sample. Each set of samples underwent MAE for a total period of 60 min, with sequential recovery and analysis of the extraction solvent after each irradiation interval (5, 10, 15, 20, 30, 45 or 60 min). Relative recovery was measured by comparison of the total RR/m, summed over all extraction intervals, for each solvent volume examined. KET and NKET were detected in these bone samples, while DHNK was not. For KET, extraction was complete within 15 min using 5 mL of ACE:HEX, while use of 10 mL extraction solvent resulted in a longer total extraction time (20 min). For NKET, the extraction time required was the same for both solvent volumes (10 min). For both

KET and NKET, there was no significant difference in total recovery between the different solvent volumes examined ($p>0.05$, U-test). Figure 6 illustrates the extraction profiles for KET and NKET in 5 and 10 mL extraction solvent following microwave irradiation.

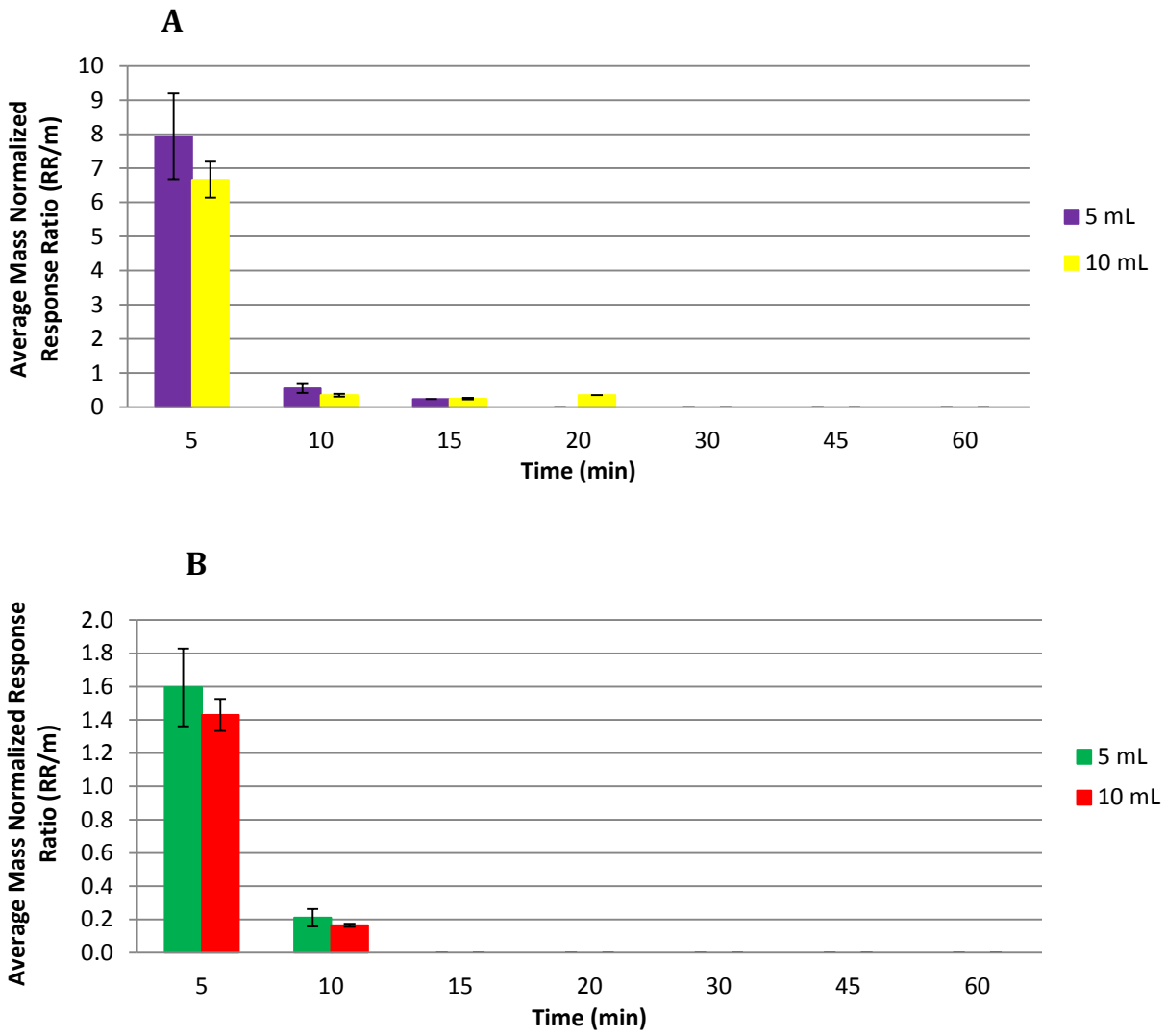

Figure 6: Extraction profile of (A) ketamine and (B) norketamine from 0.5 g bone, in various volumes of ACE:HEX (1:1, v/v) following microwave irradiation. Recovery is expressed as the mass-normalized response ratio (RR/m), which is directly proportional to concentration. Error bars represent standard deviation.

2.3.6 Passive Solvent Extraction

Different volumes of ACE:HEX and methanol were examined, to determine their influence on the extraction time required and net recovery. The total analysis time was 180 min with

sequential recovery at various time points (5, 10, 15, 20, 30, 45, 60, 90 or 180 min). KET and NKET were detected in these bone samples, but DHNK was not. The majority of KET was extracted in the first 5 min for both solvents. KET had additional recovery up to 180 min. NKET was completely recovered in the first five minutes. There was no statistical difference in total recovery ($p<0.05$) between PSE and MAE for KET and NKET for each ACE:HEX and methanol volume examined. Figure 7 shows the extraction profile for KET and NKET in both solvents. Figure 8 shows the net recovery of each analyte undergoing PSE and MAE for each ACE:HEX volume examined.

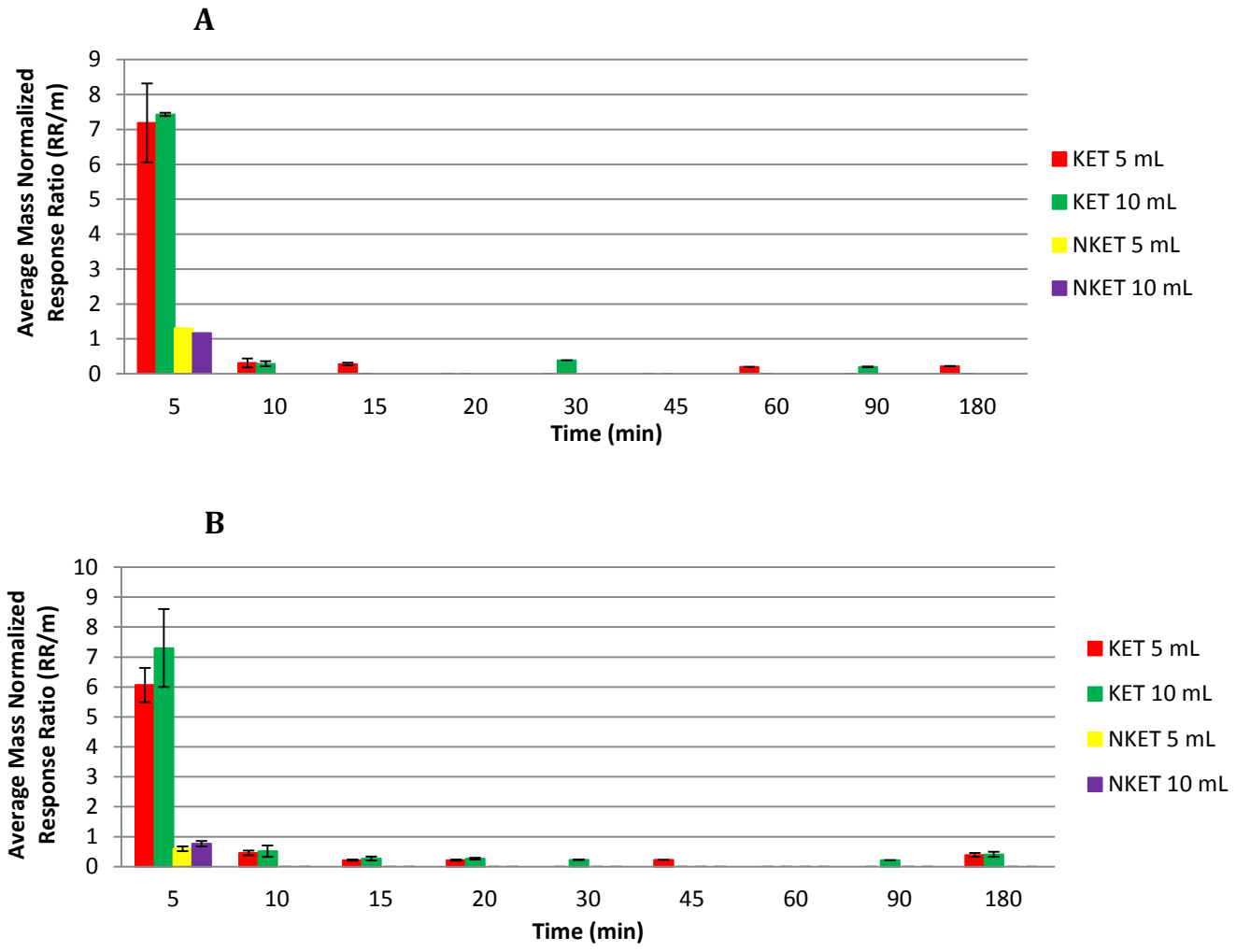

Figure 7: Extraction profile of KET and NKET in (A) methanol and (B) acetone-hexane (1:1, v/v) from 0.5 g bone, in various volumes of ACE:HEX (1:1, v/v) following passive solvent extraction. Recovery is expressed as the mass-normalized response ratio (RR/m), which is directly proportional to concentration. Error bars represent standard deviation.

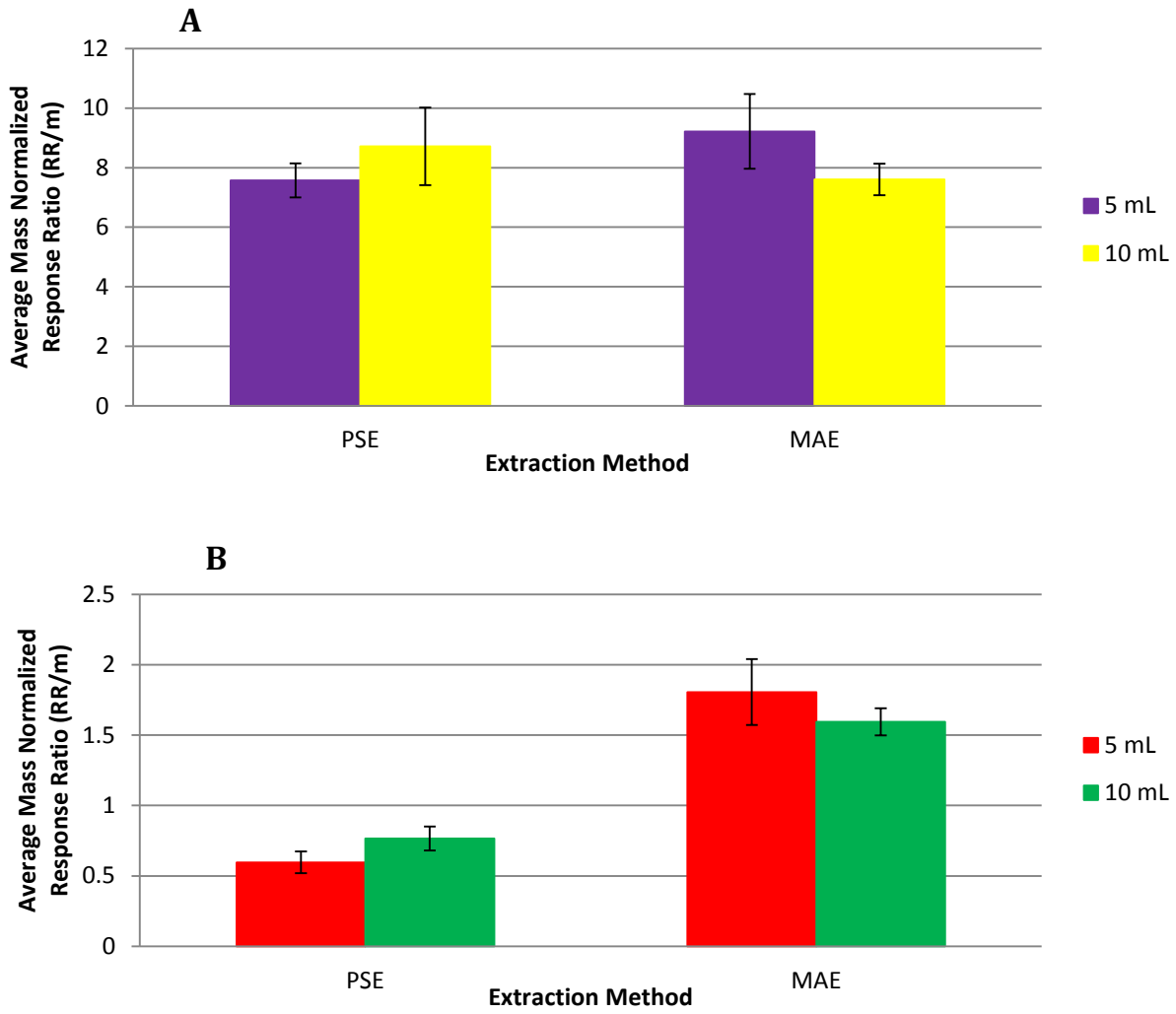

Figure 8: The net recovery of (A) KET and (B) NKET from 0.5 g bone in different volumes of ACE:HEX as a function of the method of extraction. Recovery is expressed as the mass-normalized response ratio (RR/m), which is directly proportional to concentration. Error bars represent standard deviation.

2.3.7 PSE – Bone Particle Size Comparison

Two different grinding systems were examined to evaluate their effect on total extraction yield. Each set of samples underwent PSE on a hotplate for a total period of 3 hours. KET and NKET were detected in these bone samples but DHNK was not. There was no statistical difference in total recovery ($p>0.05$) between household coffee grinder and the laboratory grade micromill-ball mill system for each volume examined. Figure 9 shows the net recovery of each analyte for both grinding systems and methanol volumes examined.

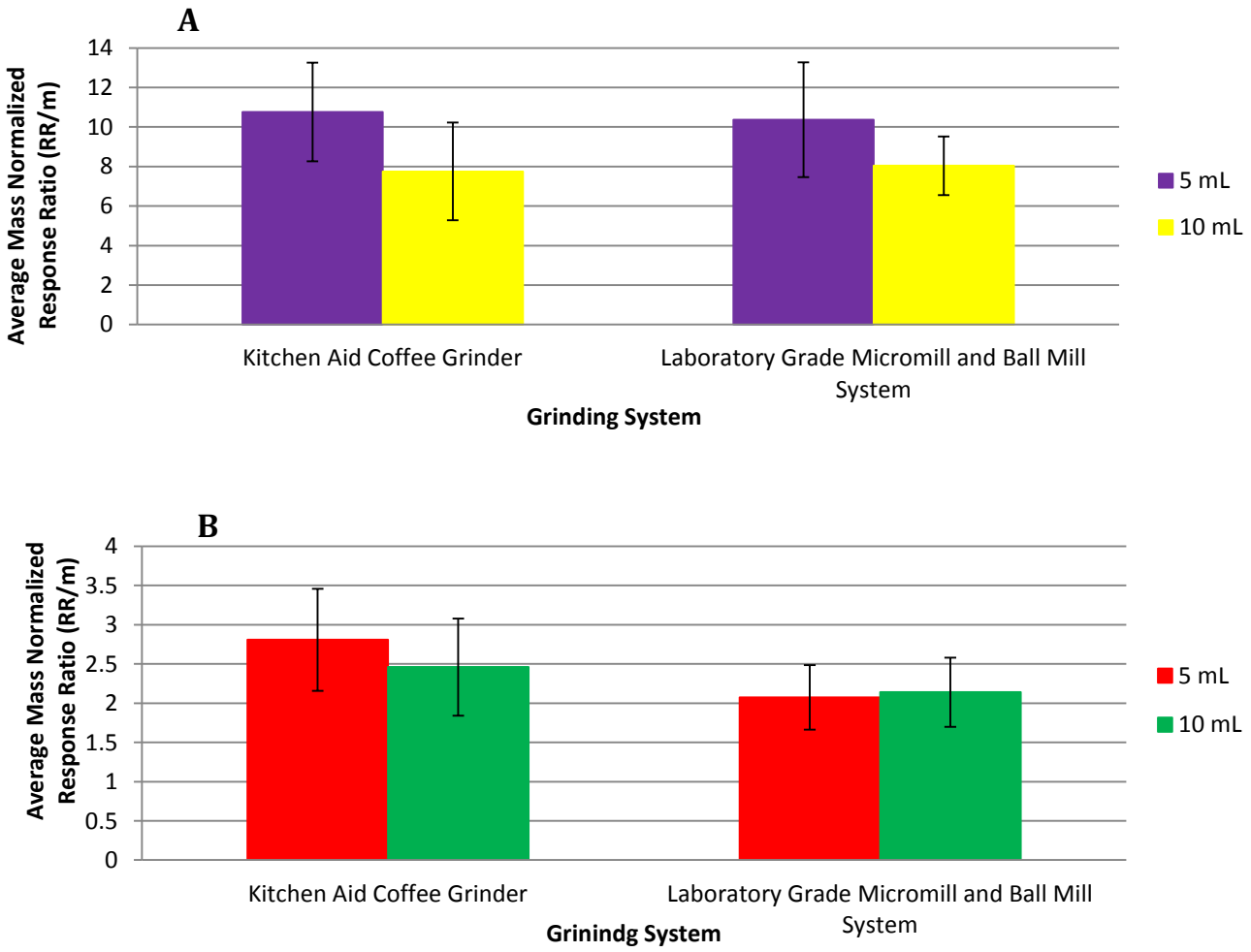

Figure 9: The net recovery of (A) KET and (B) NKET from 0.5 g bone in different volumes of methanol as a function of the grinding system. Recovery is expressed as the mass-normalized response ratio (RR/m), which is directly proportional to concentration. Error bars represent standard deviation.

2.4 Discussion

2.4.1 Implications of Experimental Design and Results

Skeletal tissues remain amongst the least understood biological matrices for toxicological analysis, although bone may be the only option for such analysis in death investigations involving severely decomposed remains. The processes by which drugs and metabolites distribute into, and are liberated from, bone are complex and vary depending on the anatomic site

of collection, the type of bone (cortical vs. trabecular) which influences the degree of vascularization and type of marrow (red vs. yellow) which is contained within, and the nature of the decomposition process (temperature, humidity, entomological activity, scavenging, etc.). The widely variable distribution of drug and metabolite concentrations between different bones in a given body (Watterson et al., 2010, Desrosiers et al., 2012, Watterson et al., 2012), in combination with these other processes, implies that a large database of bone tissue drug and metabolite measurements is necessary to develop models that may be useful to discriminate between different patterns of toxin exposure. Control of experimental environment and other variables such as dose and drug use history, in addition to providing for sufficient replication, requires that animal models be used in this work. As sample preparation is the most laborious aspect of toxicological analysis of bone, our laboratory is currently working towards streamlining of sample preparation processes to optimize analytical throughput. In this study, some experimental factors (extraction solvent, irradiation time and solvent volume) in microwave assisted extraction of ketamine and metabolites from ground bone were examined and compared to passive solvent extraction. Accordingly, a variety of bone types were collected, pooled and mixed thoroughly after pulverization in order to create as homogeneous a sample as possible for analytical method development and characterization.

2.4.2 Stability of the Drugs in Microwave Exposure

The effect of microwave irradiation was investigated for each standard drug solution, prepared in methanol or ACE:HEX, for 30 min and 60 min compared to samples that did not undergo microwave irradiation. The results of these experiments, displayed in figures 2 and 3, show that, in the case of methanol, the analytical response displayed a steadily decreasing trend for all three analytes. This result was somewhat surprising and the fate of the analytes under those conditions (i.e., chemical transformation, adsorptive loss, etc.) is not known at this time. Conversely, the analytes displayed an initial apparent increase in analytical response after the first 30 min of irradiation in ACE:HEX. While the small amount of solvent evaporation observed could not account for this change, the measurements after 60 min were in good agreement with analyte response prior to irradiation, so that no evidence of analyte loss was apparent with this extraction solvent system. Hence future work involving MAE of KET and its metabolites from bone will make use of ACE:HEX as the extraction solvent.

2.4.3 Extraction Parameters

Figures 5 to 7 show that use of 5 mL extraction solvent with a 0.5 g sample of bone provided a good balance between analyte recovery and minimization of solvent use for both MAE and PSE. No improvement in analyte recovery was observed when 10 mL extraction solvent was used, and there was a slight increase in reaction time required in MAE, presumably due to the increase in thermal mass with the larger solvent volume. While the data might appear to suggest that KET was extracted more slowly than NKET, it is important to note that the extraction time required will be dependent to a large degree upon the analyte loading in a given bone sample. In this work, bones were obtained from rats that were euthanized shortly after drug exposure, resulting in a substantial excess of KET relative to NKET. Overall, the parameters examined here would yield successful drug recovery. Extraction methods should be developed separately for each solvent or solvent systems examined. As well, the extraction time should be as long as possible to obtain maximal drug recovery. In MAE, KET took at least 20 min to maximally extract from the bone samples; we feel that an overall 30 min extraction time at 80˚C is optimal. The additional 10 min of extraction time was added to allow more concentrated samples ample time to extract. In PSE, KET was maximally extracted in 180 min so we suggest that at least 180 min extraction time. The extraction temperature in PSE cannot exceed the boiling point of the solvent so a longer period is required for extraction. The bone analyzed here was pooled from a range of different bone types. This effectively dilutes the bone, relative to samples derived from sites known to have significantly higher analyte levels, such a vertebral or pelvic bone (Watterson et al., 2010, Watterson et al., 2011, Watterson et al., 2012). Sample size should be investigated as an additional extraction parameter in a case-by-case basis. With small animal models, 0.5 g is a reasonable sample size and constitutes the entirety of certain bone types (e.g., humerus). Tissues from humans or larger animals may be sampled in higher masses (>1-2 g), and may then require adjustment of extraction time to ensure maximum yields.

2.4.4 PSE vs. MAE: Total Extraction Recovery

Figure 8 shows the total recovery for KET and NKET using both extraction methods. These data show a tendency towards higher extraction recovery in MAE compared to PSE. KET showed higher or similar recoveries using MAE compared to PSE. NKET showed higher recoveries with MAE. Based upon this data, MAE appeared to be giving a better total recovery. It is important

to remember that the benefits of MAE will depend upon the drug in question; however the results shown here indicate that MAE should be considered for the extraction of drugs from bone samples.

2.4.5 Bone Particle Size Comparison

Figure 9 shows the total recovery for KET and NKET using both grinding systems. The data shows that there is similar recovery between the two extraction methods for both grinding systems. The laboratory grade micromill-ball mill system produced a bone powder while the Kitchen Aid coffee grinder produced small chunks. It would be expected then, that the powder would produce a higher recovery due to its increased surface area. For the samples ground with the Kitchen Aid grinder, the large chunks were cortical bone and the powder was trabecular bone which absorbs the drug to a greater degree than the cortical bone (Watterson et al., 2008). Furthermore, if the drugs accumulate closer to the surface of the bone, further pulverizing will only expose the inner portions of the bone, which has accumulated a smaller degree of drug.

2.4.6 Non-detection of DHNK

As mentioned previously, one purpose of this work was to validate a method for the analysis of DHNK in bone samples. The validation data show that DHNK can be detected in bone tissue extracts with concentrations as low as 25 ng/mL. However, in the drug positive bone samples examined, DHNK was not detected. For this particular study, the bone samples examined were taken from a large mass that contained various types of bones. Because of this, the bone drug levels are diluted as drug distribution within bones depends upon the anatomical site (Watterson et al., 2008, 2010, 2011, 2012). Furthermore, DHNK is a minor metabolite of KET and in order for it to be produced, NKET must undergo degradation. The rats in this study were given an acute dose and with the half-life of KET being approximately 40 min in rats (Williams et al., 2004); there was little time for NKET accumulation, which would, in turn, limit the amount of DHNK formed by metabolized.

2.4.7 Benefits of MAE

MAE provides a significant advantage in terms of extraction time and may improve overall drug recovery. The extraction methods reported to date, most by passive extraction or acid digestion, have used lengthy extraction times, ranging from 3 to 72 hours (McIntyre et al., 2000,

Gorczynski et al., 2001, Horak et al., 2005, McGrath et al., 2009, Desrosiers et al., 2012, Watterson et al, 2012,). Microwave assisted extraction has the ability to reduce extractions to as little as 5 minutes. However, this depends largely on the drug in question, choice of solvent, the amount of solvent used and the mass of tissue sampled. To date, most work in our laboratory has used extended passive methanolic extraction (Watterson et al., 2008, 2010, 2011, 2012), where 24-72 hours was allowed for analyte extraction. In this work, maximum drug extraction was observed in 20 minutes for each of the conditions examined. Throughput was further enhanced with the microwave configuration used here by means of sample stirring and the capacity for simultaneous extraction of up to 40 samples.

2.4.8 Conclusion

The data presented here suggests that MAE may be an efficient methodology for toxicological analyses involving skeletal tissues. The optimum extraction procedure for KET and its metabolites required 30 min of microwave radiation in an acetone:hexane solvent mixture (1:1, v/v). Overall, experimental parameters such as extraction solvent, solvent volume and time should be optimized for each compound of interest as stability may vary. The extraction behaviour of drugs and their metabolites as well as the ratio of parent drug and a given metabolite, may serve as an indicator of pattern of drug exposure (i.e. acute vs. repeated), or time delay between drug exposure and death. This methodology is currently being applied to studies of the influence of body position and microclimate on ketamine and metabolite levels in various bone types.

Chapter 3

3 The Influence of Body Position and Microclimate on Ketamine and Metabolite Distribution in Decomposed Skeletal Remains

The influence of body position and microclimate on ketamine (KET) and metabolite distribution in decomposed bone tissue was examined. Rats received 75 mg/kg (i.p.) ketamine (n=30) or remained drug-free (controls, n=4). Following euthanasia, rats were divided into two groups and placed outdoors to decompose in one of three positions: supine (SUP), prone (PRO) or upright (UPR). One group decomposed in a shaded, wooded microclimate (site 1) while the other decomposed in an exposed sunlit microclimate with gravel substrate (site 2), roughly 500 m from site 1. Following decomposition, bones (lumbar vertebrae, thoracic vertebra, cervical vertebrae, rib, pelvis, femora, tibiae¸ humeri and scapulae) were collected and sorted for analysis. Clean, ground bones underwent Microwave Assisted Extraction (MAE) using acetone: hexane mixture (1:1, v/v), followed by solid phase extraction and analysis using GC/MS. Drug levels, expressed as mass normalized response ratios, were compared across all bone types between body position and microclimates. Bone type was a main effect ($p<0.05$) for drug level and drug/metabolite level ratio for all body positions and microclimates examined. Microclimate and body position significantly influenced observed drug levels: higher levels were observed in carcasses decomposing in direct sunlight, where reduced entomological activity led to slowed decomposition.

3.1 Introduction

The use of bone as a toxicological specimen may occur in cases where blood, urine or liver samples are unavailable for analysis due to advanced decomposition. There have been numerous studies involving the detection of the drugs in bone tissue (McIntyre et al., 2000, Raikos et al., 2001, Horak et al., 2005, McGrath et al., 2009, Watterson et al., 2010, 2011). These studies have used a variety of different skeletal tissues for analysis, and some only examined a small subset of tissues. Recent studies in our laboratory have examined a wide range of drugs (administered to rats under a range of conditions) in various skeletal tissues following decomposition (Lafreniere et al., 2009, Watterson et al., 2010, 2011, 2012, 2013). The analysis of these tissues showed

tissue dependence for the observed levels with highest levels generally observed in the central cavity (i.e. vertebrae, pelvis etc.) and lower levels observed in the humeri and scapulae. This distribution may be the result of differing amounts of trabecular bone content, different amounts of marrow in direct contact with the bone, or different degrees of drug adsorption from decompositional fluids following tissue liquefaction in the putrefaction process. To date, there have been no studies investigating the influence of body position on the distribution of drugs in skeletal tissues after decomposition. If drug adsorption from decompositional fluids is one mechanism of drug deposition in bone, examination of drug levels in bone recovered from different body positions during decomposition may provide valuable insight as to where drug accumulation occurs under different conditions and provide further evidence for the ideal bone types for analysis.

The effects of post-mortem environments on drug levels in skeletal tissues are also poorly understood. A small number of studies have examined assay response to bone tissue during burial (Gorczynski et al., 2001, Raikos et al., 2001, Guillot et al., 2007). They observed a difference in drug levels between samples that underwent burial and samples that did not. Midazolam, morphine, and 6-acetylmorphine showed higher drug levels in skeletal tissues not having undergone burial (Gorczynski et al., 2001, Raikos et al., 2001, Guillot et al., 2007). Another study showed burial effects to be drug and tissue dependent with Ketamine showing significant difference in observed levels while Diazepam did not (Desrosiers et al., 2010). No studies have examined the effect of differences in microclimate (i.e., differences in localized climatic conditions across short geographic distances) on detection of drugs in decomposed skeletal tissues. General decomposition studies have differing opinions as to which causes a faster decay rate, shaded areas (Galloway et al., 1999, Sharanowski et al., 2008) or sun-exposed areas (Shean et al., 1993, Majola et al., 2013). All of these studies showed a strong correlation between the decay rate and maggot activity (Galloway et al., 1999, Sharanowski et al., 2008, Shean et al., 1993, Majola et al., 2013). Studies have shown that maggots absorb and metabolize drugs from the substrates on which they feed and that concentrations of drugs detected in maggots were reflective of the total dose infused into that substrate (Miller et al., 1994, Gunn et al., 2006, Gosselin et al., 2010). These are important factors to consider in understanding the implications and limitations of using bone tissue for toxicological analyses.

In this study, differences in microclimate (i.e., differences in shade/sun exposure and surrounding flora over small geographic distances) and body position were investigated to determine their influence on ketamine (KET), norketamine (NKET) and dehydronorketamine (DHNK) distribution in decomposed skeletal tissues. Rats used in this study were given an acute dose of KET. Rats were divided into two groups and placed in a shaded/wooded or exposed/sunlit microclimate to determine if microclimate had a significant effect on the observed drug levels. Within each microclimate, rat carcasses were placed in one of three positions to decompose (supine, prone or upright), and examined to investigate if significant differences in observed bone drug levels were present. Bones underwent Microwave Assisted Extraction (MAE), solid phase extraction and were subsequently analyzed using gas chromatography-mass spectrometry, using methodology recently published (Cornthwaite et al., 2014).

3.2 Methods and Materials

3.2.1 Chemicals

Methanol used in the drug extraction was HPLC grade and obtained from EMD Chemicals (Gibbstown, NJ). KET and NKET (Cerillant, Round Rock, Texas) were obtained as 1.0 mg/mL methanolic solutions and diluted as required. KET-d4 and NKET-d4 (Cerillant, Round Rock, Texas) were obtained as 100 µg/mL methanolic solutions and diluted as required. DHNK (Cerillant, Round Rock, Texas) was obtained as a 100 µg/mL acetonitrile solution and diluted as required. Acetonitrile used was reagent grade and obtained from J.T. Baker (Center Valley, PA). All other chemicals were reagent grade and obtained from EMD chemicals.

3.2.2 Animal Administration and Study Sites

Male Wistar rats (n=30), weighing between 200 and 300 grams, were donated by Dr. T.C. Tai of the Northern Ontario School of Medicine. The rats received an acute dose of ketamine (75 mg/kg, i.p.) and euthanized by CO_2 asphyxiation within 20 minutes of drug administration. This dose was chosen as it is an anaesthetic dose in rats. Post-mortem, the rats were divided into two groups (n=15 per group), each of which was further sub-divided into three groups based upon the position in which carcasses would be arranged during decomposition: supine - SUP (n=5), prone - PRO (n=5) and upright - UPR (n=5). Two drug free rats were also used as negative controls at each site. Each group of rats was placed outdoors at one of sites and allowed to

decompose to skeleton during the summer (Late June -July) with full exposure to weather features (sunlight, precipitation etc.). The two sites, one shaded/wooded with grassy/soily ground with extensive tree cover (site 1) and the other unshaded, with high direct sun exposure and gravel substrate (no underlying soil), situated on an elevated, rocky terrain with minimal vegetation (site 2) were located on the campus of Laurentian University in Sudbury, Ontario, Canada. At both sites, animals decomposing in the supine and prone positions were placed underneath wire mesh while animals in the upright position were set on pegs in cages for the duration of decomposition. Images of set-up and decomposition for both sites can be seen in appendices C and D. Site-specific climatic data was not recorded however daily average temperature and relative humidity readings were recorded and ranged from 10-27 ^{o}C and 59-93%, respectively (data located in appendix B). Weather and the presence of insect activity were observed and noted. The carcasses at the shaded microclimate took 1 week to skeletonize while the carcasses at the unshaded microclimate took 4 weeks to decompose. After decomposition, bones collected for analysis included lumbar vertebrae (LVRT), thoracic vertebra (TVRT), cervical vertebra (CVRT) , ribs (RIB), pelvis (PEL), tibiae (TIB), femora (FEM), scapulae (SCAP) and humeri (HUM).

3.2.3 Bone Preparation and Microwave Assisted Extraction

Bones were separated according to type (e.g. femur, pelvis) and source animal (e.g. SUP-Animal 1, UPR- Animal 4). Bones were washed sequentially with phosphate buffer (0.1M, pH 6.0), methanol and acetone to remove any loosely bound soil and other contaminants. Bones were left out to air-dry for 48 hours. Bones were ground using a two part system. First, a Micro-Mill (Bel-Air Products, Pequannock, NJ) was used for 2min followed by a Ball Mill (SPEX Sample Prep., Metuchen, NJ) for 1min. Pulverized bone samples (0.5g) were weighed into PTFE-lined extraction vessels and 5mL of extraction solvent acetone:hexane (Ace:Hex, 1:1 v/v) was added. A laboratory grade microwave was used for the extractions with independent stirring in each of the 40 vessels to ensure constant sample agitation throughout the extraction. Extractions were performed by MARS 6 (CEM Corp., USA) at 80˚C for 30min. Prior to solvent removal, samples were cooled for 20min. After the extraction period, samples were washed with 5mL of extraction solvent and pooled with the original fraction. Samples were evaporated to dryness under a gentle stream of air at 70˚C, on a heating block, and reconstituted in 1mL PB6 (0.1 M, pH 6.0).

3.2.4 Solid Phase Extraction

To each sample, internal standards [200ng KET-d4 and 200ng NKET-d4], 100µL glacial acetic acid and 3 mL acetonitrile: methanol (1:1 v/v) was added. Samples were stored at -20˚C for 12h to precipitate proteins and lipids. The samples were centrifuged (4000 rpm) for 10 min and the supernatant was collected and evaporated to 1mL under a gentle stream of air (70˚C). The samples were diluted to 4 mL with PB6 (0.1M, pH 6.0) and acidified with 100µL glacial acetic acid.

Solid phase extraction was performed using CleanScreen XCEL 1 48 Well Plates (130 mg, United Chemical Technologies, Bristol, PA). Columns were first conditioned with 3mL methanol, 3 mL distilled water and 3 mL PB6. Samples were loaded on the columns and allowed to flow by gravity. The columns were washed with 3 mL PB6 and 3 mL 0.1 M acetic acid. The columns were dried using a vacuum pump for 5 minutes at 400mmHg. After drying, the columns were rinsed with 3 mL methanol. The columns were dried using a vacuum pump for 10 minutes at 400mmHg. The samples were eluted with 4mL 80:17:3 ethyl acetate/isopropanol/ammonium hydroxide. Extracts were then evaporated to dryness under a gentle stream of air (70°C) and reconstituted in 100 µL ethyl acetate for analysis by gas chromatography–mass spectrometry (GC–MS).

3.2.5 Gas Chromatography Mass Spectrometry

Extracts were analyzed on PerkinElmer Clarus 600C GC–MS (PerkinElmer LAS, Shelton, CT) using electron impact ionization, in the selected ion monitoring (SIM) mode. Separation was achieved using a ZB-Drug-1 column (30 m × 0.25 mm × 0.25 µm, Phenomenex, Torrance, CA). Extracts (2 µL) were injected into the injector, which was maintained at 250°C. The GC oven temperature was initially set to 60°C and held constant for 3 min. The set temperature was directly increased to 170°C at a rate of 130°C/min and then increased to 205°C at a rate of 2°C/min. The oven temperature was then set to directly increase to 300°C at a rate of 60°C/min and held at that temperature for 3 min at the end of the analytical run. The total GC–MS run time was 26 min. The ions monitored were *m/z* 180, 182, and 209 for KET (underlined ion used for quantitative analysis), *m/z* 166, 168, and 195 for NKET, *m/z* 153, 138 and 221 for DHNK, *m/z* 170, 172 and 199 for NKET-d4 and *m/z* 184, 213, and 156 for KET-d4. The response ratio (RR) is defined as the ratio of peak area for the quantitation ion (*m/z* 180, 166 or 153) to that of the ion

with *m/z* 184 or 170 corresponding to KET-d4 or NKET-d4. NKET-d4 was used as the internal standard for DHNK.

3.2.6 Method Validation

Samples of drug-free decomposed bone were sonicated in PB6 (100g in 500mL), after decomposition outdoors, to prepare the drug free matrix used in method validation. Samples were prepared in 1 mL of the calibrant at concentrations varying from 0 to 10000 ng/mL for KET and NKET, and 0 to 2000 ng/mL for DHNK in replicate analysis (n=3). They were subjected to the same steps (described above) as the MAE extracts drug-free and KET-exposed rats. The limits of detection and quantification were 10 ng/mL for KET and NKET and 25 ng/mL for DHNK. Precision was measured in triplicate analysis across 3 days (%coefficient of variation, %CV of the ratio of measured responses of m/z 180(KET), 166(NKET), DHNK (153), KET-d4 (184) and NKET-d4 (170) respectively) ranged from 2 to 19%. This assay was shown to be linear for KET (R^2 = 0.9934), NKET (R^2 = 0.9954) and DHNK (R^2 = 0.9973). A sample of ethyl acetate was run following each concentration in one standard curve to evaluate the degree of carryover. At the all concentrations, the carryover was less than 10% of the smallest calibrator (10 ng/mL). Due to the presence of carryover, a sample of ethyl acetate was run after each drug positive sample.

3.2.7 Gas Chromatography Mass Spectrometry Autosampler Stability

The stability of the GC-MS response to KET and its metabolites was examined after the samples sat on the autosampler. Samples of KET, NKET and DHNK were prepared in 1 mL of bone tissue extract at two different concentrations, 100 ng/mL and 1000 ng/mL in triplicate. Samples underwent the same extraction procedure as described above, and were analyzed every six hours for a total of 48 hours while situated in the autosampler. For all analytes at both concentration levels examined, the analytical response remained within ±20% of initial values, indicating that samples were stable on the autosampler for up to 48 hr.

3.2.8 Statistical Analysis

All statistical analysis was performed using StatPlus 2009 software (AnalystSoft Inc.,www.analystsoft.com). The Mann-Whitney U-test, a non-parametric alternative to the t-test, was used to compare measured values (RR/m KET, NKET and DHNK) between different

environmental sites. The Kruskall-Wallis test, a non-parametric analysis of variance, was used to compare measured values (RR/m KET, NKET and DHNK) within one site across different decomposition positions. These nonparametric tests were chosen as they do not presume that measured values within a group are normally distributed.

3.3 Results

3.3.1 Expression of Drug Levels

In this work, we report the drug levels as the mass normalized response ratios (RR/m) for KET, NKET and DHNK measurements. This has been described in a variety of published studies (Watterson et al., 2011, 2012, 2013) and is done because analyte recovery from solid bone tissue cannot be accurately measured or calibrated for using techniques conventional to forensic toxicology (i.e., GC-MS or LC-MS). Proper calibration of analyte recovery is not possible because the solid bone sample is heterogeneous and cannot be homogenously mixed with a drug standard for purposes of recovery measurements. However, the RR/m value is proportional to concentration and allows the comparison of drug levels between different experimental treatments using similarly prepared samples. These values can be converted to estimates of bone drug or metabolite concentrations, however they should only be used as approximations due to the reasons stated above.

3.3.2 Influence of Body Position on Drug Distribution

The influence of body position on KET, NKET and DHNK levels in decomposed skeletal remains was examined for each microclimate. KET and NKET were detected in all bone types examined at each microclimate. The average measured drug levels (i.e., RR/m) of KET and NKET are illustrated in Figures 10 and 11. The Kruskall-Wallis (KW) test was performed to determine whether bone type or body position during decomposition was a main effect for the observed drug levels of KET, NKET, DHNK as well as the ratio of levels of KET and NKET (RR_{KET}/RR_{NKET}), ratio of levels of KET and DHNK (RR_{KET}/RR_{DHNK}) and ratio of levels of NKET and DHNK (RR_{NKET}/RR_{DHNK}) within each site. Kruskall-Wallis analysis showed that bone type was a main effect for levels of KET, NKET and (RR_{KET}/RR_{NKET}). For KET and NKET analyses, the body position during decomposition had a significant effect on the levels observed ($p<0.05$), except for the LVRT and HUM of site 2 for KET levels. The greatest KET

and NKET levels were generally observed in the SUP position for site 1 (shaded), whereas PRO and UPR positions were generally associated with higher drug levels for site 2 (sunlit), but drug levels appeared to be more similar between body positions at this site. Examination of the ratio of levels of KET and NKET (RR_{KET}/RR_{NKET}) for each bone sample also showed that the body position during decomposition was a main effect on observed values of (RR_{KET}/RR_{NKET}), ($p<0.05$), except in PEL of site 2. Average values for (RR_{KET}/RR_{NKET}) for each bone type are summarized in Figure 3 for each microclimate examined.

DHNK was detected in only two bone types examined, lumbar vertebra and thoracic vertebra in both microclimates and average measured drug levels are summarized in Figure 9 and 10. KW analysis showed that bone type was a main effect for DHNK levels observed as well as the ratio of levels of KET and DHNK (RR_{KET}/RR_{DHNK}) and the ratio of levels of NKET and DHNK (RR_{NKET}/RR_{DHNK}). For DHNK analyses, as well as the analyses of the ratio of levels of KET and DHNK (RR_{KET}/RR_{DHNK}) and the ratio of levels of NKET and DHNK (RR_{NKET}/RR_{DHNK}), KW analysis showed that the body position during decomposing had a significant effect on the levels observed ($p<0.05$) except in TVRT in site 2 for the ratio of levels of NKET and DHNK (RR_{NKET}/RR_{DHNK}).

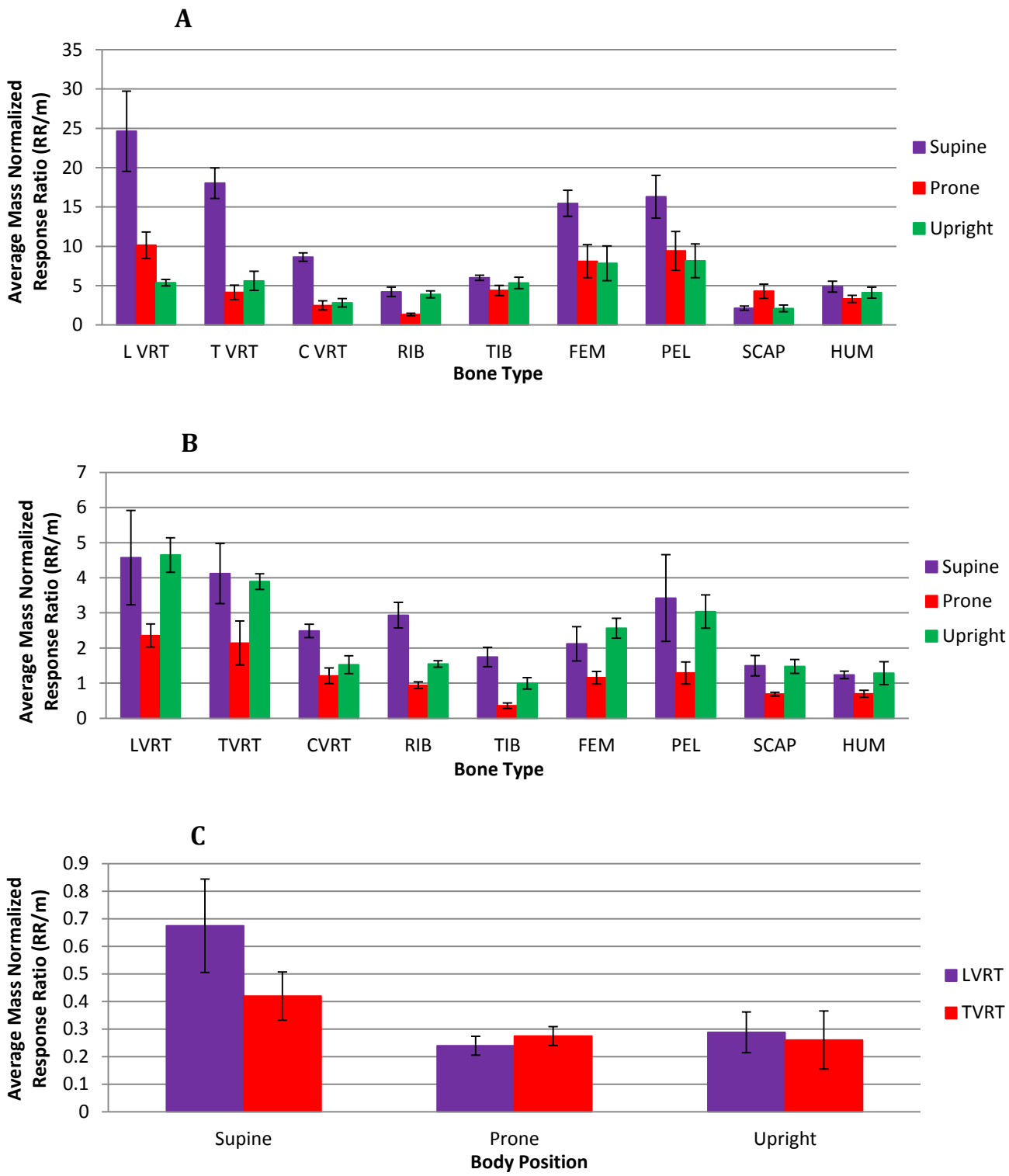

Figure 10: Average Mass Normalized Response Ratios (RR/m) of Distribution by Body Position for (A) Ketamine, (B) Norketamine and (C) Dehydronorketamine at the shaded microclimate. Error bars represent standard deviation.

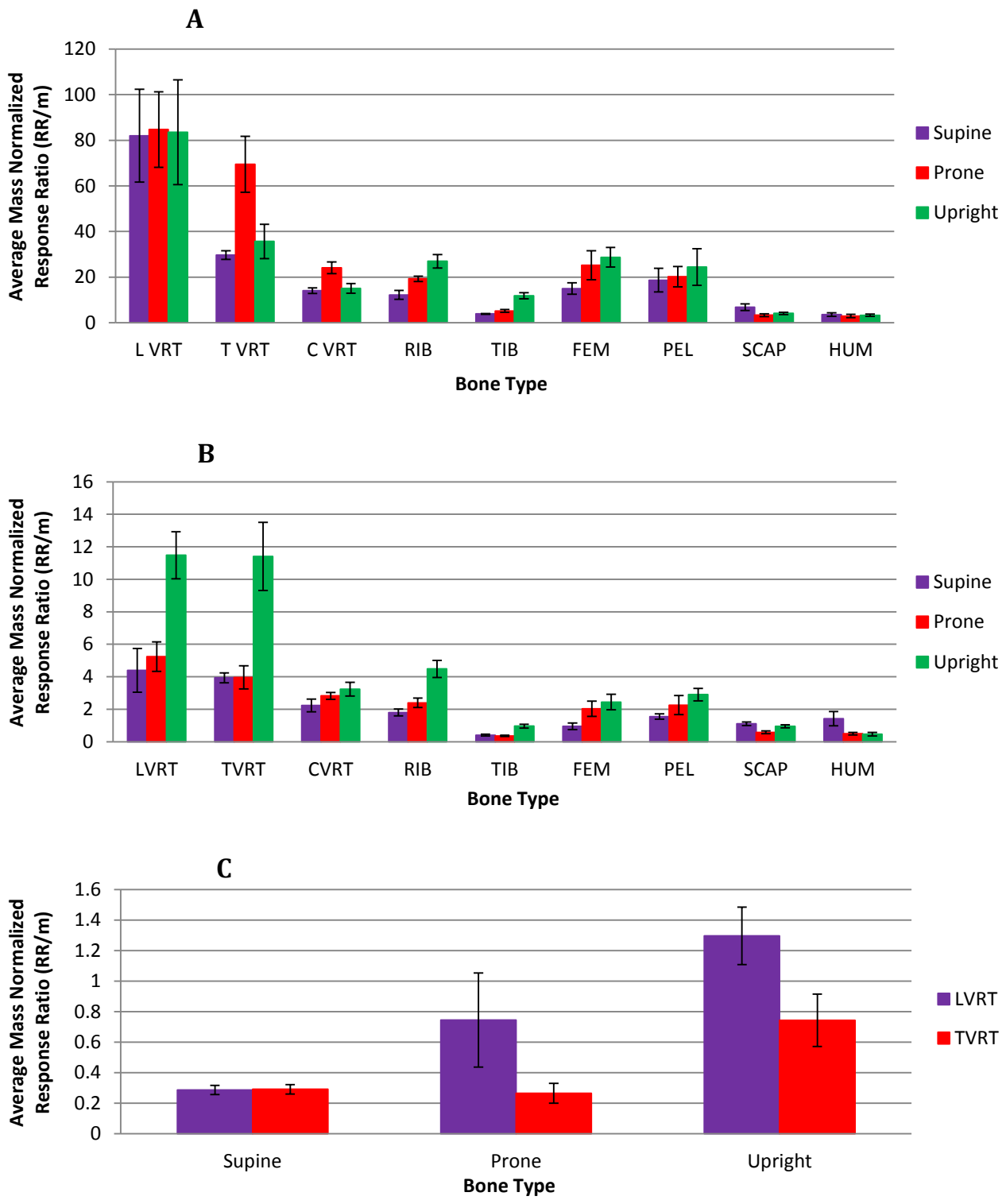

Figure 11: Average Mass Normalized Response Ratios (RR/m) of Distribution by Body Position for (A) Ketamine, (B) Norketamine and (C) Dehydronorketamine at the sunlit microclimate. Error bars represent standard deviation.

3.3.3 Influence of Microclimate on Drug Distribution

The Mann-Whitney test was used to assess differences in drug levels (RR/m) in each bone type between environments. For KET analyses, observed drug levels were significantly different ($p<0.05$) between environments in 7/9, 7/9 and 8/9 tissue types examined for SUP, PRO and UPR positions respectively with the greatest values generally observed in carcasses that decomposed in direct sunlight (site 2). For NKET analyses, observed drug levels were significantly different between environments in 5/9, 6/9 and 9/9 tissue types examined for SUP, PRO and UPR positions respectively with the greatest value, once again, in carcasses that decomposed in direct sunlight at site 2. Values of (RR_{KET}/RR_{NKET}) were significantly different between environments in 9/9, 6/9 and 9/9 tissues types examined for SUP, PRO and UPR positions respectively.

DHNK analyses showed that drug levels were significantly different between sites in 2/2, 1/2 and 2/2 tissue types examined for SUP, PRO, and UPR positions respectively, with the greatest SUP values observed in carcasses that decomposed in the shaded microclimate. The greatest PRO and UPR values were observed in carcasses that decomposed in direct sunlight. The ratio of levels of KET and DHNK and the ratio of levels of NKET and DHNK were significantly different in both tissue types examined, for all positions.

Examination of data across all tissue types examined for each microclimate showed that drug levels varied 28-fold, 23-fold and 15-fold for KET, NKET and (RR_{KET}/RR_{NKET}), respectively for site 1 (wooded/shaded), and 56-fold, 43-fold and 20-fold for KET, NKET and (RR_{KET}/RR_{NKET}), respectively for site 2 (exposed/sunlit). The drug levels for DHNK, (RR_{KET}/RR_{DHNK}) and (RR_{NKET}/RR_{DHNK}) varied 8-fold, 25-fold and 3-fold respectively. Figure 12 shows box-and-whisker plots depicting KET, NKET and (RR_{KET}/RR_{NKET}) levels corresponding to each microclimate.

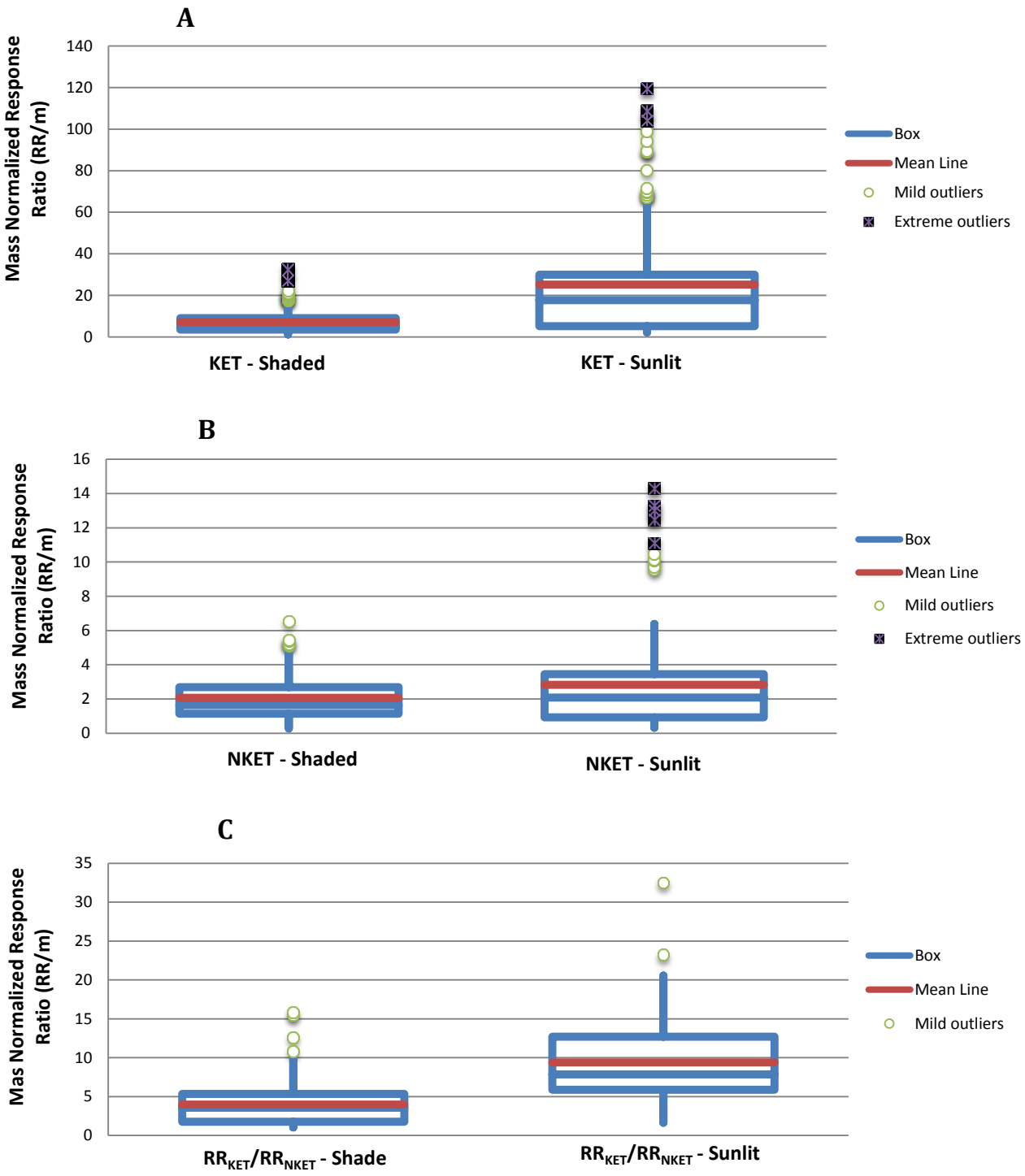

Figure 12: Summary of pooled data for shaded and sunlit microclimates. Box-and-whisker plots for pooled bone samples for mass normalized response ratios (RR/m) for (A) KET, (B) NKET and (C) the ratio of levels of KET and NKET (RR_{KET}/RR_{NKET}).

3.4 Discussion

This study is the first to examine the influence of body position and microclimate on skeletal drug distribution in following decomposition. Studies preceding this work have shown that toxicological analysis of bone is complex, although a dose-dependent response has been shown to exist in bone tissue (Watterson et al., 2011, 2012, 2013) although observed drug levels may be skewed as a result of decomposition (Lafreniere et al., 2009, Desrosiers et al., 2010). If drug adsorption from decompositional fluids is one mechanism of drug deposition in bone, it is important to examine whether environmental conditions that influence decomposition have any significant effect bone drug measurements. As decomposition proceeds, soft tissues liquefy which may result in drug partitioning from these tissues to the surrounding bone due to its somewhat porous nature. The amount of drug adsorption could be related to the surface area of contact between drug-rich fluid and the surrounding bone, the concentration of drug in decaying tissues (including marrow) and the amount of cortical or trabecular bone. Further, the position in which a body decomposed may potentially be influential as the effects of gravity would be expected to influence the contact between decompositional fluid and solid bone.

3.4.1 Implications of Body Position Data

Tissue type was a main effect for all drug level as well as the ratio of levels of drug and metabolite (RR_{KET}/RR_{NKET}, RR_{KET}/RR_{DHNK} and RR_{NKET}/RR_{DHNK}) in all cases. Figures 10 and 11 summarize the average measured drug levels for KET, NKET and DHNK. Examination of KET and NKET levels across all bone types and all positions indicated that the highest levels were observed in SUP for the carcasses that decomposed in a shaded area. This was also the case for the ratio of levels of KET and NKET. In the SUP carcasses, pooling of the fluid from the liquefying tissues was observed in the central cavity which would provide more contact with the bone surface in the posterior regions of the body. The UPR carcasses began to sag in the first two days. As a result, the fluid from the liquefying tissues would have more contact with the ground then bone tissue. Interestingly, examination of Figure 10 shows that there is a shifting of the bone type with maximum drug levels from the vertebrae (as seen in the SUP samples) toward the pelvis and femur (as seen in the UPR samples). The highest KET and NKET levels were observed in UPR for carcasses that decomposed in direct sunlight. The UPR carcasses were the

most dehydrated. This provided the greatest degree of contact between the liquefying tissues and the bone surface.

The highest KET and NKET levels for each body position were the LVRT except in the case of KET in UPR carcasses in shaded microclimate which had higher PEL values. This lends some support to the notion that liquefying tissues may be one mechanism of drug transport into bone tissue. Vertebral bone is a likely candidate to absorb drug due to its high surface area and spongy, trabecular bone. In UPR, pooling would occur at the base of the carcass, femoral and pelvic regions would account for higher drug levels in these regions.

DHNK was only detected in two out of the nine bone types examined, LVRT and TVRT. The extent of drug transfer to mineralized bone depends on multiple factors including: surface area, type of marrow and type of bone. Vertebral bone has a large surface area, red bone marrow, which is highly vascularized and is composed mainly of spongy bone. These facilitate a greater degree of mass transfer between the marrow and the mineralized bone. For these reasons, it is the type of bone mostly to absorb drugs and would provide detection of drugs with a lower concentration. The highest levels for DHNK, the ratio of levels of KET and DHNK and the ratio of levels of NKET and DHNK were similar to all drug levels; highest levels SUP for carcasses in shaded microclimate and UPR for carcasses in direct sunlight. The highest levels in each body position were TVRT for the carcasses in the shaded microclimate and LVRT for the carcasses in the direct sunlight microclimate.

3.4.2 Implicates of Microclimate Data

Microclimate significantly influenced levels for KET and NKET in 7/9 and 5/9, 7/9 and 6/9 and 8/9 and 9/9 tissue types examined for SUP, PRO and UPR respectively. The ratio of levels of KET and NKET were significantly influenced by microclimate in 9/9, 6/9 and 9/9 tissue types examined for SUP, PRO and UPR respectively. DHNK was significantly influenced by in 5/6 lumbar and thoracic vertebra samples examined for the three positions. The ratio of levels of KET and DHNK and the ratio of levels of NKET and DHNK were significantly influenced by microclimate in all tissues types examined. In the cases of microclimate influence, the higher levels were generally found in carcasses that had been exposed to direct sunlight during decomposition. These remains were largely mummified, with a much lesser degree of

entomological activity to drive decomposition. Previous studies have suggested that dehydrating conditions at site 2 (direct sunlight and low humidity) are unsuitable for maggot activity (Galloway et al., 1999, Sharanowski et al., 2008). Others studies have shown that direct sunlight may act as a catalyst for stimulating maggot activity however in these cases, the carcasses were in environments with both high heat and high humidity (Shean et al., 1993, Majola et al., 2013). The slower rate of decomposition may have led to higher measured drug levels as a result of lesser drainage of decompositional fluids from the body and lower consumption of drug-laden tissues via insect activity. The shaded carcasses had a much greater degree of maggot activity and subsequently a much faster rate of decomposition. Maggots have been shown to absorb and metabolize drugs (Miller et al., 1994, Gunn et al., 2006, Gosselin et al., 2010) which may account for the lower levels detected in the shaded carcasses. Also, these carcasses decomposed to skeleton in one week. As a result, there was little time for the liquefying tissues to interact with the bone surface and allow for drug adsorption to the bone surface.

Overall, the wide variability in the data presented in this study and others (Watterson et al., 2011, 2013) suggests that there may be limited interpretive value in quantitative measurements of individual drugs or metabolites. Without a large reference database encompassing drug levels from a variety of bone types and standardized methodologies, toxicological analysis of bone tissues remains best presented as a qualitative assessment. In this work we sought to examine the variability in individual drug and metabolite measurements as well as the ratio of levels of parent drug and metabolite, in order to determine if parent-metabolite ratio measurements were able to offset variability in drug or metabolite levels arising from differences in body position or microclimate during the decomposition process. Table I summarizes the variability, expressed as the ratio of maximum to minimum RR/m values (MAX/MIN), within a given bone type, across an animal and within a body position. As can be seen by examining the MAX/MIN values, variability in (RR_{KET}/RR_{NKET}) was comparable to that in levels of KET and NKET across an animal or within a position. However, in examining all bone measurements from each microclimate (Figure 4), (RR_{KET}/RR_{NKET}) values were less variable than either KET or NKET levels (MAX/MIN values of 28, 23 and 15, respectively, at site 1, and 56, 43 and 20, respectively, at site 2). Another important observation, consistent with what has been observed in previous work (Watterson et al., 2011, 2013) is that the relative variability within a bone type (i.e., across different animals) was generally lower than that observed across bones within an

animal, suggesting that drug level from one tissue type should be compared to a database of drug levels from that same tissue type if any interpretive value is to be gleaned.

Table 6: Ratio of Maximum and Minimum Drug Levels (RR/m) For Ketamine and Norketamine at Each Body Position and Ratio of Maximum to Minimum Drug Level Ratios for Ketamine and Norketamine (RRKET/RRNKET) at Each Body Position for (A) Shaded Microclimate and (B) Direct Sunlight Microclimate.

A

Body Position	Max/Min Range Within Given Bone Type	Max/Min Range Within Animal	Max/Min Pooled Bone and All Animals
Ketamine (KET)			
Supine	1.2-1.6	9-17	18
Prone	1.3-2.0	6-10	11
Upright	1.2-1.9	3-6	6
Norketamine (NKET)			
Supine	1.2-2.3	3-5	6
Prone	1.2-1.9	4-9	10
Upright	1.2-1.8	3-6	7
KET/NKET			
Supine	1.1-2.5	3-10	10
Prone	1.2-1.9	7-10	13
Upright	1.2-2.4	4-6	6

B

Body Position	Max/Min Range Within Given Bone Type	Max/Min Range Within Animal	Max/Min Pooled Bone and All Animals
Ketamine (KET)			
Supine	1.1-2.2	17-31	45
Prone	1.2-1.9	21-47	49
Upright	1.3-2.0	16-33	44
Norketamine (NKET)			
Supine	1.2-1.9	9-14	19
Prone	1.2-1.8	9-16	19
Upright	1.3-1.8	16-40	40
KET/NKET			
Supine	1.2-2.4	4-20	20
Prone	1.2-2.1	3-4	5
Upright	1.2-2.0	4-6	6

3.4.3 Conclusion

This is the first environmental study that examined skeletal drug distribution under different influences of microclimate and body position during the decomposition process. First, drug distribution was found to vary between different body positions, potentially due to variation of the degree of contact between liquefying tissues and bone surface. Second, microclimatic differences (e.g., direct sunlight, humidity, temperature, etc.) that may affect the entomological activity around the remains are associated with variations in drug and metabolite distribution in bone. Similar studies should to be performed using other drugs to assess the general applicability of the data presented here.

Chapter 4

4.1 General Conclusions

A high throughput MAE and MPSPE protocol was developed for detection and quantification of KET and its two metabolites, NKET and DHNK. Analytes were extracted from bone in 30 minutes using MAE with a similar recovery to PSE, which required 180 minutes for extraction. Secondly, the net recovery of analytes was similar for both grinding systems. Furthermore, microplate solid phase extraction proved to be a more efficient configuration then the block manifold. Using this protocol, influences of body position and microclimate were examined as they related to distribution in decomposed skeletal tissues. Observed KET and NKET levels were significantly different between body positions with the highest levels found in supine positioned rats for the shaded microclimate and upright positioned rats for the sunlit microclimate. In terms of microclimate, the sunlit region had much higher drug levels with substantially less maggot activity. Important to note, DHNK was only detected in two bone types: LVRT and TVRT. This may be attributed to the dose administered.

4.2 Future Work

Skeletal tissues are one of the least understood biological matrices available in forensic toxicology so further research is required. Generalized d rug extractions for classes of drugs should be developed. The research presented here, examines microclimatic differences that may affect drug distribution in decomposed skeletal tissues and is the first such study conducted in the forensic toxicology research lab (FTRL). This study was performed on a small scale to accumulate preliminary data. This type of study should be applied to a wide variety of drugs to access whether or not similar trends exist and on a larger scale. Furthermore, additional microclimates should be investigated. This study looked specifically at terrestrial decomposition however examination of aquatic decomposition and burial would provide a more complete decomposition profile. A partially shaded microclimate would be worth investigating to determine if the decomposition resembles the shaded or sunlit microclimates. For this particular study, temperature, humidity and rain fall data was collected from a weather network. In future studies, site specific and internal carcass temperature data should also be collected to correlate between sites and with weather networks. The season in which carcasses decompose should also

be considered. Finally, maggots, flies and beetles should be collected to determine their degree of influence on the observed drug levels.

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Appendices

Appendix A: Images of Bone Prepared with the Grinding Systems

Figure A1: Bone Prepared with the Kitchen Aid Coffee Grinder

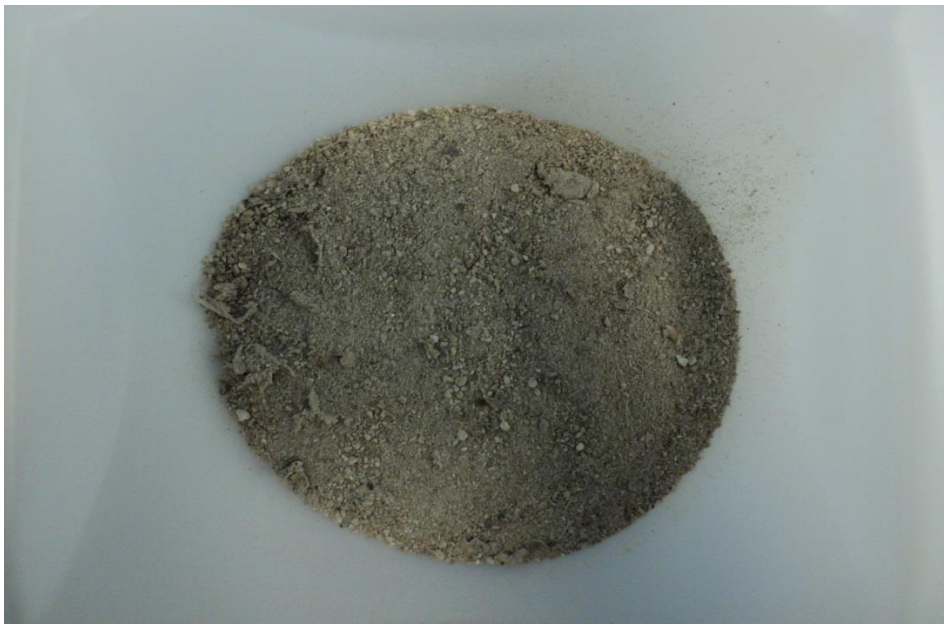

Figure A2: Bone Prepared with the Micro-mill/Ball Mill Grinding System

Appendix B: Temperature Data for Environmental Study

Date	Min Temp (˚C)	Max Temp (˚C)	Avg. Temp (˚C)	Humidity (%)	Weather
15-Jul-13	16	31	24	66	Sunny with Cloudy Patches
16-Jul-13	17	33	25	67	Sunny
17-Jul-13	21	33	27	78	Sunny
18-Jul-13	18	27	22	85	Cloudy, Thunderstorm
19-Jul-13	19	26	22	91	Cloudy, Rain
20-Jul-13	13	21	17	68	Sunny
21-Jul-13	6	21	14	62	Sunny with Cloudy Patches
22-Jul-13	9	25	17	64	Sunny with Cloudy Patches
23-Jul-13	9	23	16	77	Overcast, Mostly Cloudy
24-Jul-13	6	21	14	64	Sunny
25-Jul-13	9	23	16	69	Sunny
26-Jul-13	11	23	17	69	Cloudy, Rain
27-Jul-13	14	21	18	85	Overcast, Light Rain
28-Jul-13	12	20	17	85	Cloudy, Rain
29-Jul-13	9	19	14	87	Sunny
30-Jul-13	8	24	16	78	Sunny
31-Jul-13	12	24	18	84	Cloud Cover, Overcast
01-Aug-13	13	22	18	87	Overcast, Rain
02-Aug-13	11	20	16	93	Cloudy, Rain
03-Aug-13	9	20	14	87	Sunny
04-Aug-13	9	21	15	66	Cloud Cover
05-Aug-13	7	21	14	69	Cloud Cover
06-Aug-13	12	21	16	86	Overcast, Light Rain
07-Aug-13	15	23	19	92	Cloudy, Rain
08-Aug-13	12	22	17	77	Sunny
09-Aug-13	9	19	14	84	Cloudy, Rain
10-Aug-13	5	22	14	78	Sunny
11-Aug-13	8	23	16	77	Cloudy, Rain
12-Aug-13	10	22	16	86	Cloudy, Rain
13-Aug-13	9	11	10	93	Sunny, Rain
14-Aug-13	7	19	13	79	Cloud Cover
15-Aug-13	8	22	15	73	Cloudy, Rain
16-Aug-13	10	23	16	71	Sunny
17-Aug-13	12	25	18	65	Sunny

Appendix C: Images from Shaded Microclimate


Figure C1: Overall images of the shaded microclimate


Figure C2: Set-up of rats in the supine position and degree of decomposition after 1 week

Figure C3: Set-up of rats in the prone position and degree of decomposition after 1 week

Figure C4: Set-up of rats in the upright position and degree of decomposition after 1 week

Appendix D: Images from Sunlit Microclimate

Figure D1: Overall images of the sunlit microclimate.

Figure D2: Set-up of rats in the supine position and degree of decomposition after 4 weeks.

Figure D3: Set-up of rats in the prone position and degree of decomposition after 4 weeks.

Figure D4: Set-up of rats in the upright position and degree of decomposition after 4 weeks